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(57) Abstract

A vaccine composition is disclosed that comprises polypeptides and fragments of polypeptides containing histidine triad residues or coiled–coil regions, some of which polypeptides or fragments lie between 80 and 680 residues in length. Also disclosed are processes for preventing infection caused by *S. pneumoniae* comprising administering of vaccine compositions.

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STREPTOCOCCUS PNEUMONIAE PROTEINS AND IMMUNOGENIC FRAGMENTS FOR VACCINES

This application is based on U.S. Provisional Application No. 60/113,048, filed 21 December 1998, which is hereby incorporated in its entirety.

FIELD OF THE INVENTION

This invention relates generally to the field of bacterial antigens and their use, for example, as immunogenic agents in humans and animals to stimulate an immune response. More specifically, it relates to the vaccination of mammalian species with a polypeptide comprising at least one conserved histidine triad residue (HxxHxH) and at least one helix-forming polypeptide obtained from *Streptococcus pneumoniae* as a mechanism for stimulating production of antibodies that protect the vaccine recipient against infection by a wide range of serotypes of pathogenic *S. pneumoniae*. Further, the invention relates to antibodies against such polypeptides useful in diagnosis and passive immune therapy with respect to diagnosing and treating such pneumococcal infections.

In a particular aspect, the present invention relates to the prevention and treatment of pneumococcal infections such as infections of the middle ear, nasopharynx, lung and bronchial areas, blood, CSF, and the like, that are caused by pneumococcal bacteria.

BACKGROUND OF THE INVENTION

Streptococcus pneumoniae is a gram positive bacteria which is a major causative agent in invasive infections in animals and humans, such as sepsis, meningitis, otitis media and lobar pneumonia (Tuomanen et al. *New Engl. J. Med.* **322**:1280-1284 (1995)). As part of the infective process, pneumococci readily bind to non-inflamed human epithelial cells of the upper and lower respiratory tract by binding to eukaryotic carbohydrates in a lectin-like manner (Cundell et al., *Micro. Path.* **17**:361-374 (1994)). Conversion to invasive pneumococcal infections for bound bacteria may involve the local generation of inflammatory factors which may activate the epithelial cells to change the number and type of receptors on their surface (Cundell et al., *Nature*, **377**:435-438 (1995)). Apparently, one such receptor, platelet activating factor (PAF) is engaged by the pneumococcal bacteria and within a very short period of time (minutes) from the appearance of PAF, pneumococci exhibit strongly enhanced adherence and invasion of tissue. Certain soluble receptor analogs have been shown to prevent the progression of pneumococcal infections (Idanpaan-Heikkila et al., *J. Inf. Dis.*, **176**:704-712 (1997)). A number of various other proteins have been suggested as being involved in the pathogenicity of *S. pneumoniae*. There remains a need for identifying polypeptides having epitopes in common from various strains of *S. pneumoniae* in order to utilize such polypeptides as vaccines to provide protection against a wide variety of *S. pneumoniae*.

SUMMARY OF INVENTION

In accordance with the present invention, there is provided vaccines and

vaccine compositions that include polypeptides obtained from *S. pneumoniae* and/or variants of said polypeptides and/or active fragments of such polypeptides.

The active fragments, as hereinafter defined, include a histidine triad residue(s) and/or coiled coil regions of such polypeptides.

The term "percent identity" or "percent identical," when referring to a sequence, means that a sequence is compared to a claimed or described sequence from an alignment of the sequence to be compared (the "Compared Sequence") with the described or claimed sequence (the "Reference Sequence"). The percent identity is determined as follows:

$$\text{Percent Identity} = [1 - (C/R)]\ 100$$

wherein C is the number of differences between the Reference Sequence and the Compared Sequence over the length of the alignment between the Compared Sequence and the Reference Sequence wherein (i) each base or amino acid in the Reference Sequence that does not have an aligned base or amino acid in the Compared Sequence and (ii) each gap in the Reference Sequence and (iii) each aligned base or amino acid in the Reference Sequence that is different from an aligned base or amino acid in the Compared Sequence, each being a difference; and R is the number of bases or amino acids in the Reference Sequence over the length of the alignment with the Compared Sequence with any gap created in the Reference Sequence also being counted as a base or amino acid.

If an alignment exists between the Compared Sequence and the Reference Sequence in which the Percent Identity as calculated above is about

equal to or greater than a specified minimum Percent Identity than the Compared Sequence has the specified minimum Percent Identity to the Reference Sequence even though alignments may exist in which the hereinabove calculated Percent Identity is less than the specified Percent Identity.

"Isolated" in the context of the present invention with respect to polypeptides and/or polynucleotides means that the material is removed from its original environment (*e.g.*, the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living organism is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the co-existing materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment. The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

BRIEF DESCRIPTION OF DRAWINGS

Figures 1A-1C, respectively, report the results of three experiments using different preparations of SP36. The results demonstrate that active immunization with recombinant SP36 derived from pneumococcal strain Norway serotype 4 is able to protect mice from death in a model of pneumococcal sepsis using a heterologous strain, SJ2 (serotype 6B). In each of the three experiments shown, one hundred percent of the mice immunized

with SP36 survived for the 14-day observation period following challenge with approximately 500 cfu of pneumococci, while eighty to one hundred percent of sham-immunized mice (injected with PBS and adjuvant) died during the same period.

Figures 2A-2B show that passive administration of rabbit antiserum raised against Sp36 derived from Norway type 4 was able to protect mice in the pneumococcal sepsis model using two heterologous strains. Figure 2A shows that one hundred percent of the mice immunized with the SP36 antiserum survived the 21-day observation period after challenge with 172 CFU of strain SJ2 (serotype 6B). Eighty percent of the mice immunized with a control serum (rabbit anti-FimC) died by day 8, and ninety percent died by day 12. Figure 2B shows that 90 percent of the mice immunized with the Sp36 antiserum survived the 8-day observation after challenge with 862 CFU of strain EF6796 (serotype 6A). Ninety percent of the mice immunized with a control serum (collected before immunization) died by day 5.

Figure 3 is a western blot demonstrating the ability of antisera raised against recombinant Sp36 derived from strain Norway type 4 to react with Sp36 of heterologous strains. Total cell lysates were immunoblotted with mouse antisera to Sp36. A band representing Sp36 protein was detected in all 23 *S. pneumoniae* strains tested, which included isolates from each of the 23 pneumococcal serotypes represented in the current polysaccharide vaccine.

Figure 4 is a Southern blot showing that the Sp36 gene from Norway type 4 hybridizes with genomic DNA from 24 other pneumococcal strains, indicating the presence of similar sequences in all these strains.

Figure 5 is a western blot showing the reactivity of patient sera with Sp36. Sp36 (either full-length, panel A; N-terminal half, panel B; or C-terminal half, panel C) was electrophoresed by SDS-PAGE and transferred to nitrocellulose. Patient sera collected soon after the onset of illness (acute serum, lanes A) or eight to 30 days later (convalescent serum, lanes C) were used to probe the blots. For patients 2, 3, and 5, convalescent serum reacted more strongly with Sp36 than did the corresponding acute serum.

Figure 6 is an amino acid alignment comparison of four related pneumococcal proteins, namely Sp36A (PhtA; SEQ ID NO:8), Sp36B (PhtB; SEQ ID NO:10), Sp36D (PhtD; SEQ ID NO:4), Sp36E (PhtE; SEQ ID NO:6), respectively. Dashes in a sequence indicate gaps introduced to maximize the sequence similarity. Amino acid residues that match are boxed.

Figure 7 is a nucleotide alignment comparison of four related pneumococcal genes, namely Sp36A (PhtA; SEQ ID NO:9), Sp36B (PhtB; SEQ ID NO:11), Sp36D (PhtD; SEQ ID NO:5), Sp36E (PhtE; SEQ ID NO:7), respectively. Dashes in a sequence indicate gaps introduced to maximize the sequence similarity.

Figure 8 shows the results of immunization of mice with PhtD recombinant protein, which leads to protection from lethal sepsis. C3H/HeJ (Panel A and B) or Balb/cByJ (Panel C) mice were immunized subcutaneously with PhtD protein (15 μg in 50 μl PBS emulsified in 50 μl complete Freund's adjuvant (CFA)). The recombinant PhtD protein used in protection experiments consisted of 819 amino acid residues, starting with the cysteine

(residue 20). A group of 10 sham-immunized mice received PBS with adjuvant. A second immunization of 15 μg protein with incomplete Freund's adjuvant (IFA) was administered 3 weeks later; the sham group received PBS with IFA. Blood was drawn (retro-orbital bleed) at week 7; and sera from each group was pooled for analysis of anti-PhtD antibody by ELISA. Mice were challenged at week 8 by an intraperitonial (i.p.) injection of approximately 550 CFU *S. pneumoniae* strain SJ2, serotype 6B (Panel A), 850 CFU of strain EF6796, serotype 6A (Panel B) or 450 CFU of strain EF5668, serotype 4 (Panel C). In preliminary experiments, the LD_{50} for strain SJ2 and EF6796 were determined to be approximately 10 CFU for both strains. The LD_{50} for strain EF5668 was determined to be < 5 CFU. Survival was determined in all groups over the course of 15 days following challenge. Data are presented as the percent survival for a total of 10 mice per experimental group. Two-sample Log-rank test was used for statistical analysis comparing recombinant Pht immunized mice to sham-immunized mice.

SUMMARY OF THE INVENTION

In accordance with one aspect of the present invention, there is provided a vaccine, generally in the form of a composition, that includes at least one polypeptide that is at least 90% identical to (c) a polypeptide

sequence comprising amino acids 1-819 of SEQ ID NO:4 or (ii) a polypeptide sequence comprising amino acids 1-460 of SEQ ID NO:6 or an active fragment of the foregoing.

In accordance with another aspect of the present invention, there is provided a vaccine, generally in the form of a composition, that includes an active fragment of a polypeptide that is at least 90% identical to (i) a polypeptide comprising amino acids 1-800 of SEQ ID NO:8 or (ii) a polypeptide comprising amino acids 1-800 of SEQ ID NO:10.

The term "active fragment" means a fragment that includes one or more histidine triad residues and/or one or more coiled coil regions. A "histidine triad residue" is the portion of the polypeptide that has the sequence HxxHxH wherein H is histidine and x is an amino acid other than histidine

A coiled coil region is the region predicted by "Coils" algorithm: Lupas, A., Van Dyke, M., and Stock, J. (1991) Predicting Coiled Coils from Protein Sequences, *Science* **252**:1162-1164.

In accordance with one embodiment, the active fragment includes both one or more histidine triad residues and at least one coiled coil region of the applicable polypeptide sequence. In accordance with another embodiment, the active fragment includes at least two histidine triad residues.

In another embodiment, the active fragment that includes at least one histidine triad residue or at least one coiled-coil region of the applicable polypeptide includes at least about ten percent of the applicable polypeptide and no more than about 85% of the applicable polypeptide.

The polypeptide of SEQ ID NO:4 includes five histidine triad residues, as follows:

amino acids 64-69; 188-193; 296-301; 541-546; and 625-630.

The polypeptide of SEQ ID NO:6 includes five histidine triad residues, as follows:

amino acids 63-68; 185-190; 289-294, 376-381; and 441-446.

In addition, the polypeptide of SEQ ID NO:4 includes two coiled-coil regions (amino acids 120-140 and amino acids 750-772) and the polypeptide of SEQ ID NO:6 includes one coiled-coil region (amino acids 119-152).

The polypeptide of SEQ ID NO: 8 includes the following regions:

HxxHxH: amino acids 63-68, 189-194, 309-314, 550-555, 634-639.
Coiled-coils: amino acids 118-145, 406-434, 462-493, 724-751.

In accordance with a further aspect of the invention, a vaccine of the type hereinabove described is administered for the purpose of preventing or treating infection caused by *S. pneumoniae*.

A vaccine, or vaccine composition, in accordance with the present invention may include one or more of the hereinabove described polypeptides or active fragments thereof. When employing more than one polypeptide or active fragment, such two or more polypeptides and/or active fragments may be used as a physical mixture or as a fusion of two or more polypeptides or active fragments. The fusion fragment or fusion polypeptide may be produced,

for example, by recombinant techniques or by the use of appropriate linkers for fusing previously prepared polypeptides or active fragments.

In an embodiment of the invention, there is provided (a) a polypeptide that is at least 95% identical or at least 97% identical or 100% identical to (i) a polypeptide sequence comprising amino acids 1 to 819 of SEQ ID NO:4 or (ii) a polypeptide sequence comprising amino acids 1-460 of SEQ ID NO:6; or (b) an active fragment of the polypeptide of (a).

In the case where the polypeptide is a variant of the polypeptide comprising the mature polypeptide of SEQ ID NO:4 or SEQ ID NO:6, or any of the active fragments of the invention, the variation in the polypeptide or fragment is generally in a portion thereof other than the histidine triad residues and the coiled-coil region, although variations in one or more of these regions may be made.

In many cases, the variation in the polypeptide or active fragment is a conservative amino acid substitution, although other substitutions are within the scope of the invention.

In accordance with the present invention, a polypeptide variant includes variants in which one or more amino acids are substituted and/or deleted and/or inserted.

In another aspect, the invention relates to passive immunity vaccines formulated from antibodies against a polypeptide or active fragment of a polypeptide of the present invention. Such passive immunity vaccines can be utilized to prevent and/or treat pneumococcal infections in patients. In this manner, according to a further aspect of the invention, a vaccine can be

produced from a synthetic or recombinant polypeptide of the present invention or an antibody against such polypeptide.

In still another aspect the present invention relates to a method of using one or more antibodies (monoclonal, polyclonal or sera) to the polypeptides of the invention as described above for the prophylaxis and/or treatment of diseases that are caused by pneumococcal bacteria. In particular, the invention relates to a method for the prophylaxis and/or treatment of infectious diseases that are caused by *S. pneumoniae*. In a still further preferred aspect, the invention relates to a method for the prophylaxis and/or treatment of otitis media, nasopharyngeal, bronchial infections, and the like in humans by utilizing a vaccine of the present invention.

Generally, vaccines are prepared as injectables, in the form of aqueous solutions or suspensions. Vaccines in an oil base are also well known such as for inhaling. Solid forms which are dissolved or suspended prior to use may also be formulated. Pharmaceutical carriers are generally added that are compatible with the active ingredients and acceptable for pharmaceutical use. Examples of such carriers include, but are not limited to, water, saline solutions, dextrose, or glycerol. Combinations of carriers may also be used.

Vaccine compositions may further incorporate additional substances to stabilize pH, or to function as adjuvants, wetting agents. or emulsifying agents, which can serve to improve the effectiveness of the vaccine.

Vaccines are generally formulated for parental administration and are injected either subcutaneously or intramuscularly. Such vaccines can also be formulated as suppositories or for oral administration, using methods known in the art.

The amount of vaccine sufficient to confer immunity to pathogenic bacteria is determined by methods well known to those skilled in the art. This quantity will be determined based upon the characteristics of the vaccine recipient and the level of immunity required. Typically, the amount of vaccine to be administered will be determined based upon the judgment of a skilled physician. Where vaccines are administered by subcutaneous or intramuscular injection, a range of 50 to 500 μg purified protein may be given.

The present invention is also directed to a vaccine in which a polypeptide or active fragment of the present invention is delivered or administered in the form of a polynucleotide encoding the polypeptide or active fragment, whereby the polypeptide or active fragment is produced *in vivo*. The polynucleotide may be included in a suitable expression vector and combined with a pharmaceutically acceptable carrier.

In addition, the polypeptides of the present invention can be used as immunogens to stimulate the production of antibodies for use in passive immunotherapy, for use as diagnostic reagents, and for use as reagents in other processes such as affinity chromatography.

In another aspect the present invention provides polynucleotides which encode the hereinabove described polypeptides and active fragments of the invention. The polynucleotide of the present invention may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand.

In accordance with another aspect of the present invention, there is

provided

(A) an isolated polynucleotide that is at least 90% identical to a polynucleotide sequence encoding (i) a polypeptide comprising amino acids 1-819 of SEQ ID NO:4 or (ii) a polypeptide comprising amino acids 1-460 of SEQ ID NO:6, or

(B) a fragment of the polynucleotide of (A) that encodes an active polypeptide fragment or

(C) a polynucleotide that is at least 90% identical to a polynucleotide sequence encoding an active fragment of (i) a polypeptide comprising amino acids 1 to 800 of SEQ ID NO:8 or (ii) a polypeptide comprising amino acids 1 to 800 of SEQ ID NO:10.

In specific embodiments, the polynucleotide is at least 95% identical, preferably at least 97% identical, and even 100% identical to such polynucleotide sequence.

The term "polynucleotide encoding a polypeptide" encompasses a polynucleotide which includes only coding sequence for the polypeptide as well as a polynucleotide which includes additional coding and/or non-coding sequence.

The present invention further relates to variants of polynucleotides. The variants of the polynucleotides may be a naturally occurring allelic variant of the polynucleotides or a non-naturally occurring variant of the polynucleotides. The variants include variants in which one or more bases are substituted, deleted or inserted. Complements to such coding polynucleotides may be utilized to isolate polynucleotides encoding the same or similar polypeptides. In particular, such procedures are useful to obtain native immunogenic portions of polypeptides from different serotypes of *S. pneumoniae*, which is especially

useful in the production of "chain" polypeptide vaccines containing multiple immunogenic segments.

SEQ ID NO:5 is a representative example of a polynucleotide encoding the polypeptide of SEQ ID NO:4 and SEQ ID NO:7 is a representative example of a polynucleotide encoding the polypeptide of SEQ ID NO:6. SEQ ID NO:9 is a representative example of a polynucleotide encoding the polypeptide of SEQ ID NO:8, and SEQ ID NO:11 is a representative example of a polynucleotide encoding the polypeptide of SEQ ID NO:10. As a result of the known degeneracy of the genetic code, other polynucleotides that encode the polypeptides of SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8 and SEQ ID NO:10 should be apparent to those skilled in the art from the teachings herein.

The polynucleotides encoding the immunogenic polypeptides described above may also have the coding sequence fused in frame to a marker sequence which allows for purification of the polypeptides of the present invention. The marker sequence may be, for example, a hexa-histidine tag supplied by a pQE-9 vector to provide for purification of the mature polypeptides fused to the marker in the case of a bacterial host, or, for example, the marker sequence may be a hemagglutinin (HA) tag when a mammalian host, e.g. COS-7 cells, is used. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson, I., et al., Cell, 37:767 (1984)).

The present invention also relates to vectors which include polynucleotides encoding one or more of the polypeptides of the invention, host cells which are genetically engineered with vectors of the invention and the production of such immunogenic polypeptides by recombinant techniques in an isolated and substantially immunogenically pure form.

Host cells are genetically engineered (transduced or transformed or transfected) with the vectors comprising a polynucleotide encoding a polypeptide of the invention. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the polynucleotides which encode such polypeptides. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

Vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may be used as long as it is replicable and viable in the host.

The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: LTR or SV40 promoter, the E. coli. lac or trp, the phage lambda P_L promoter and other promoters known to control expression of genes in

prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in E. coli.

The vector containing the appropriate DNA sequence as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the proteins.

As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as E. coli, Streptomyces, Salmonella typhimurium; fungal cells, such as yeast; insect cells such as Drosophila S2 and Spodoptera Sf9; animal cells such as CHO, COS or Bowes melanoma; adenoviruses; plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

More particularly, the present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter,

operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example. Bacterial: pQE70, pQE60, pQE-9 (Qiagen, Inc.), pbs, pD10, phagescript, psiX174, pbluescript SK, pbsks, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene); ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia). Eukaryotic: pWLNEO, pSV2CAT, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia). However, any other plasmid or vector may be used as long as they are replicable and viable in the host.

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are pKK232-8 and pCM7. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda P_R, P_L and TRP. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Davis, L., Dibner, M., Battey, I., Basic Methods in Molecular Biology, (1986)).

The constructs in host cells can be used in a conventional manner to

produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y., (1989), the disclosure of which is hereby incorporated by reference.

Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Examples including the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of E. coli and S. cerevisiae TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), α-factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with

translation initiation and termination sequences. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include E. coli, Bacillus subtilis, Salmonella typhimurium and various species within the genera Pseudomonas, Streptomyces, and Staphylococcus, although others may also be employed as a matter of choice.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, a french press, mechanical disruption, or use of cell lysing agents, such methods are well know to those skilled in the art. However, preferred are host cells which secrete the polypeptide of the invention and permit recovery of the polypeptide from the culture media.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, Cell, 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

The polypeptides can be recovered and/or purified from recombinant cell cultures by well-known protein recovery and purification methods. Such methodology may include ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity

chromatography, hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. In this respect, chaperones may be used in such a refolding procedure. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The polypeptides that are useful as immunogens in the present invention may be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated.

Procedures for the isolation of the individually expressed polypeptides may be isolated by recombinant expression/isolation methods that are well-known in the art. Typical examples for such isolation may utilize an antibody to a conserved area of the protein or to a His tag or cleavable leader or tail that is expressed as part of the protein structure.

The polypeptides, their fragments or other derivatives, or analogs thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. These antibodies can be, for example, polyclonal or monoclonal antibodies. The present invention also includes chimeric, single chain, and humanized antibodies, as well as Fab fragments, or the product of an Fab expression library. Various procedures known in the art may be used for the production of such antibodies and fragments.

Antibodies generated against the polypeptides corresponding to a

sequence of the present invention can be obtained by direct injection of the polypeptides into an animal.

For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, 1975, Nature, 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole, et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96).

Techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce single chain antibodies to immunogenic polypeptide products of this invention. Also, transgenic mice may be used to express humanized antibodies to immunogenic polypeptide products of this invention.

The invention will be further described with respect to the following examples; however, the scope of the invention is not limited thereby:

Example 1

Active Protection with Anti-Sp36

A. Cloning, expression, and purification of SP36

The genomic DNA used as target for amplification was isolated from *S. pneumoniae* Norway strain (serotype 4), the same strain used for genomic sequencing. The complete sequence of the Sp36 gene (SEQ ID NO:9), and its predicted amino acid sequence (SEQ ID NO:8), are given in the Sequence Listing appended hereto. It was noted that the predicted amino acid

sequence included a hydrophobic leader sequence followed by a sequence (LSVC) similar to the consensus sequence for Type II signal peptidase (LxxC, in which both x's typically represent small amino acids). Primers (listed as SEQ ID NOS:1-3) were designed that would amplify the Sp36 gene and allow its cloning into pQE10 and expression as a histidine-tagged protein lacking the signal sequence for purification by nickel-affinity chromatography. Cloning of the fragment amplified by SEQ ID Nos 1 and 3 would result in a protein containing amino acids 2 through 800 of Sp36; cloning of the fragment amplified by SEQ ID Nos 2 and 3 would result in a protein containing amino acids 7 through 800 of Sp36 (amino acid numbers refer to SEQ ID NO:8).

B. Active Protection With Sp36 Vaccination

In each of the three experiments shown in Figures 1A-1C, C3H/HeJ mice (10/group) were immunized intraperitoneally (i.p.) with Sp36 protein (15 μg in 50 μl PBS emulsified in 50 μl complete Freund's adjuvant (CFA)). A group of 10 sham-immunized mice received PBS with adjuvant. A second immunization of 15 μg protein with incomplete Freund's adjuvant (IFA) was administered 4 weeks later; the sham group received PBS with IFA. Blood was drawn (retro-orbital bleed) at weeks 3, 6, and 9; and sera from each group were pooled for analysis of anti-Sp36 antibody by ELISA. Mice were challenged at week 10 by an i.p. injection of approximately 500 CFU *S. pneumoniae* strain SJ2 (serotype 6B; provided by P. Flynn, St. Jude Children's Research Hospital, Memphis, TN). In preliminary experiments, the LD_{50} of this strain was determined to be approximately 10 CFU. Mice were monitored for 14 days for survival.

The three experiments shown in Figures 1A-1C used slightly different

preparations of recombinant Sp36. The experiments shown in Figure 1A and 1B both used Sp36 containing amino acids 20-815, but different batches of protein were used in the two experiments. The experiment shown in Figure 1C used Sp36 containing amino acids 25-815.

In the experiment shown in Figure 1A, 9-week sera collected from the ten mice immunized with Sp36 (first batch) had an endpoint ELISA titer of 1:4,096,000. No anti-Sp36 antibody was detected in sera from sham-immunized mice. One hundred percent of the mice immunized with Sp36 protein survived the challenge (520 cfu of pneumococci) for 14 days. Eighty percent of sham-immunized mice were dead by day 4, and the remainder survived.

In the experiment shown in Figure 1B, 9-week sera collected from the ten mice immunized with Sp36 (second batch) had an endpoint ELISA titer of >1:4,096,000. No anti-Sp36 antibody was detected in sera from sham-immunized mice. One hundred percent of the mice immunized with Sp36 protein survived the challenge (510 cfu of pneumococci) for 14 days. Of the sham-immunized mice, eighty percent were dead by day 4, and all died by day 9.

In the experiment shown in Figure 1C, 9-week sera collected from the ten mice immunized with Sp36 (containing amino acids 25- 815) had an endpoint ELISA titer of 1:4,096,000. No anti-Sp36 antibody was detected in sera from sham-immunized mice. One hundred percent of the mice immunized with Sp36 protein survived the challenge (510 cfu of pneumococci) for 14 days. Of the sham-immunized mice, ninety percent died by day 4, and all died by day 12. These data demonstrate that immunization of mice with recombinant Sp36 proteins elicits a response capable of

protecting against systemic pneumococcal infection and death. This protection was not strain-specific: the recombinant pneumococcal protein was cloned from a serotype 4 strain, while the challenge was with a heterologous strain, SJ2 (serotype 6B).

Example 2

Passive Protection with Anti-Sp36 Antisera

A. Generation of Rabbit Immune Sera

Following collection of preimmune serum, a New Zealand White rabbit was immunized with 250 µg of Sp36 (containing amino acids 20-815) in CFA. The rabbit was given two boosts of 125 µg Sp36 in IFA on days 29 and 50 and bled on days 39 and 60. A second rabbit was immunized with a control antigen, *E. coli* FimC.

B. Passive Protection in Mice

C3H/HeJ mice (10 mice/group) were passively immunized by two i.p. injections of 100 µl of rabbit serum. The first injection was administered twenty-four hours before challenge with 172 cfu of *S. pneumoniae* strain SJ2, and the second injection was given four hours after challenge. Figure 2 shows the survival of mice after infection with two different strains of pneumococci.

Figure 2A shows that of mice injected with 172 cfu of strain SJ2 (Figure 2A), one hundred percent of the mice immunized with rabbit immune serum raised against Sp36 protein survived the 21-day observation period. Of the mice immunized with the control serum (anti-FimC), eighty percent

died by day 8, and ninety percent died by day 12. Figure 2B shows that of mice injected with 862 cfu of strain EF6796, ninety percent of the mice immunized with rabbit immune serum raised against Sp36 protein survived the 8-day observation period. Of those given a control serum (collected from a rabbit before immunization), ninety percent died by day 8.

These data indicate that the protection against pneumococcal infection resulting from immunization with Sp36 is antibody-mediated, since mice can be protected by passive transfer of serum from a hyperimmunized rabbit. As seen in the mouse active challenge experiments described above, serum directed against recombinant Sp36 protein cloned from a serotype 4 strain was protective against challenge with heterologous strains.

Example 3

Conservation of Sp36 Among Strains of *S. pneumoniae*

A. Western blotting

The 23 pneumococcal strains used in this experiment were obtained from the American Type Culture Collection (Rockville, MD) and include one isolate each of the 23 serotypes in the multivalent pneumococcal vaccine. For total cell lysates, pneumococci were grown to mid-logarithmic phase (optical density at 620 nm, 0.4 to 0.6) in 2 ml Todd-Hewitt broth with 0.5% yeast extract (Difco, Detroit, ME) at 37°C. Bacteria were harvested by centrifugation and washed twice with water. Pellets were resuspended in 200 µl lysis buffer (0.01% sodium dodecyl sulfate, 0.15 M sodium citrate and 0.1% sodium deoxycholate) and incubated at 37°C for 30 min, then diluted in an equal volume 2x SSC (0.3 M sodium chloride, 0.03 M sodium citrate). Lysates were separated by SDS-PAGE, transferred to nitrocellulose

membranes (Bio-Rad Laboratories, Hercules, CA), and probed with antibody in a standard Western blotting procedure. Sera from ten C3H/HeJ mice immunized with Sp36 (as described in Example 1) were pooled and used at a dilution of 1:3000. Bound antibody was detected with peroxidase-conjugated sheep anti- mouse IgG using the chemiluminescence kit from Amersham, Inc. (Cambridge, MA).

The mouse anti-Sp36 sera detected two major bands with apparent molecular weights of 97 and 100 kDa in all 23 pneumococcal lysates tested (shown in Figure 3). The Sp36 signals obtained from *S. pneumoniae* serotypes 1, 5, 17F and 22F were lower, indicating either that the level of Sp36 expression is reduced in these strains, or that Sp36 in these strains is antigenically different.

These data show that Sp36 is antigenically conserved among strains of the 23 pneumococcal serotypes represented in the current polysaccharide vaccine.

B. Southern blotting

Genomic DNA was prepared from each of the 23 pneumococcal strains listed in the previous section and also from strain SJ2. DNA was digested with *Pvu*II and *Bam*HI, electrophoresed in an agarose gel and transferred to a nylon membrane. A probe was prepared by amplifying the Sp36 gene from Norway type 4 DNA (as in Example 1) and labeling the amplified fragment with fluorescein by the random-priming method, using a kit from Amersham. Hybridization, washing, and exposure of film were carried out as in the protocol supplied by Amersham. Figure 4 shows that

the Sp36 probe hybridized with DNA from each of the 24 strains studied. The lane marked "M" contained DNA from lambda phage, digested with *Hin*dIII and labeled with fluorescein, as molecular weight markers.

Example 4

Immunogenicity of Sp36 in Humans

In order to determine whether Sp36 is immunogenic during human pneumococcal infection, sera from patients with culture-proven pneumococcal bacteremia were used in Western blots containing recombinant Sp36 protein. In the experiment shown in Figure 5, sera from five patients (indicated as 1 through 5) were diluted 1:3000 and used to probe blots containing full-length Sp36, the N-terminal half of Sp36 (preceding the proline-rich region), or the C-terminal half of Sp36 (following the proline-rich region). Lanes labeled A (acute) were probed with serum collected shortly after diagnosis of pneumococcal infection; lanes C (convalescent) were probed with serum collected either one month later (patients 1, 2, and 3) or eight days after the first serum collection (patients 4 and 5). For patients 2, 3 and 5, reactivity of the convalescent serum with Sp36 was stronger that that of the corresponding acute serum. The difference between the acute and convalescent sera was particularly evident for reactivity with the C-terminal half of the protein.

In additional experiments (not shown), convalescent sera from 23 patients with pneumococcal infections were tested individually for reactivity with full-length Sp36: 20 of the 23 sera were found to bind Sp36 on a Western blot.

These experiments indicate that Sp36 is recognized by the human

immune system and suggest that antibodies able to bind the Sp36 protein may be produced during natural *S. pneumoniae* infection in humans. Since the patients were infected with a variety of pneumococcal strains, these data also support the idea that Sp36 is antigenically conserved.

Example 5

Table 1 provides the percent identity between the various sequences.

Alignment of the predicted amino acid sequences of PhtA, PhtB, PhtD, and PhtE using the MEGALIGN program of Lasergene showed strong N-terminal homology with substantial divergence of the C-termini (Figure 6). The alignment of the nucleotide sequences of the same genes is shown in Figure 7. Amino acid and nucleotide sequences were compared using the identity weighting in a Lipman-Pearson pairwise alignment, in which the number of matching residues is divided by the total of matching residues plus the number of mismatched residues plus the number of residues in gaps. In the table below, the percent identity between each pair of sequences is shown at the intersection of the corresponding row and column.

Example 6

Active Protection with PhtD Vaccination.

Mice immunized with recombinant PhtD derived from strain N4 generated potent antibody titers (reciprocal endpoint titers ranging form 2,048,00 to 4,096,000). Mice immunized with PhtD were protected against death following intraperitoneal injection with either of three heterologous strains, SJ2 (serotype 6B; provided by P. Flynn, St. Jude Children's Research

Hospital, Memphis, TN), EF6796 (serotype 6A) or EF5668 (serotype 4; both strains provided by D. Briles, University of Alabama, Birmingham). In the experiment shown in Figure 8 (Panel A), all ten of the sham-immunized mice died within 10-days after challenge with virulent pneumococci (strain SJ2), while eighty percent of the PhtD-immunized mice survived the 15-day observation period. Immunization with PhtD also protected against a serotype 6A strain, EF6796 (Panel B) and a serotype 4 strain, EF5668 (Panel C). In the experiment shown in Figure 8 (Panel B), all ten of the sham-immunized mice died within 7-days after challenge with virulent pneumococci (strain EF6796), while ninety percent of the PhtD-immunized mice survived the 15-day observation period. In the experiment shown in Figure 8 (Panel C), all ten of the sham-immunized mice died within 6-days after challenge with virulent pneumoccoci (strain EF5668), while eight of nine mice immunized with PhtD survived the 15-day observation period.

Table 1. Percent Identities

Percent Identity Between Amino Acid Sequences				
	PhtA	PhtB	PhtD	PhtE
PhtA	---	66.4	63.9	49.5
PhtB		---	87.2	49.5
PhtD			---	49.8
PhtE				---

Percent Identity Between Nucleotide Sequences				
	PhtA	PhtB	PhtD	PhtE
PhtA	---	58.3	59.3	47.9
PhtB		---	86.4	47.4
PhtD			---	47.9
PhtE				---

WHAT IS CLAIMED IS:

1. A vaccine composition comprising:

(a) at least one member selected from the groups consisting of (i) a polypeptide comprising a polypeptide sequence that is at least 90% identical to amino acids 1-819 of SEQ ID NO:4; (ii) a polypeptide comprising a polypeptide sequence that is at least 90% identical to amino acids 1-460 of SEQ ID NO:6; (iii) a fragment of the polypeptide of (i) that includes at least one of a histidine triad residue or coiled-coil region; (iv) a fragment of the polypeptide of (ii) that includes at least one of a histidine triad residue or a coiled-coil region; (v) a fragment of a polypeptide that is at least 90% identical to the polypeptide sequence comprising amino acids 1-800 of SEQ ID NO:8, wherein said fragment includes at least one of a histidine triad residue or coiled-coil region wherein said fragment includes at least 80 amino acids and no more than 680 amino acids; and (vi) a fragment of a polypeptide that is at least 90% identical to the polypeptide sequence comprising amino acids 1-800 of SEQ ID NO:10, wherein said fragment includes at least one of a histidine triad residue or coiled-coil region wherein said fragment includes at least 80 amino acids and no more than 680 amino acids; and

(b) a pharmaceutically acceptable carrier.

2. A process for preventing infection caused by *S. pneumoniae* comprising:

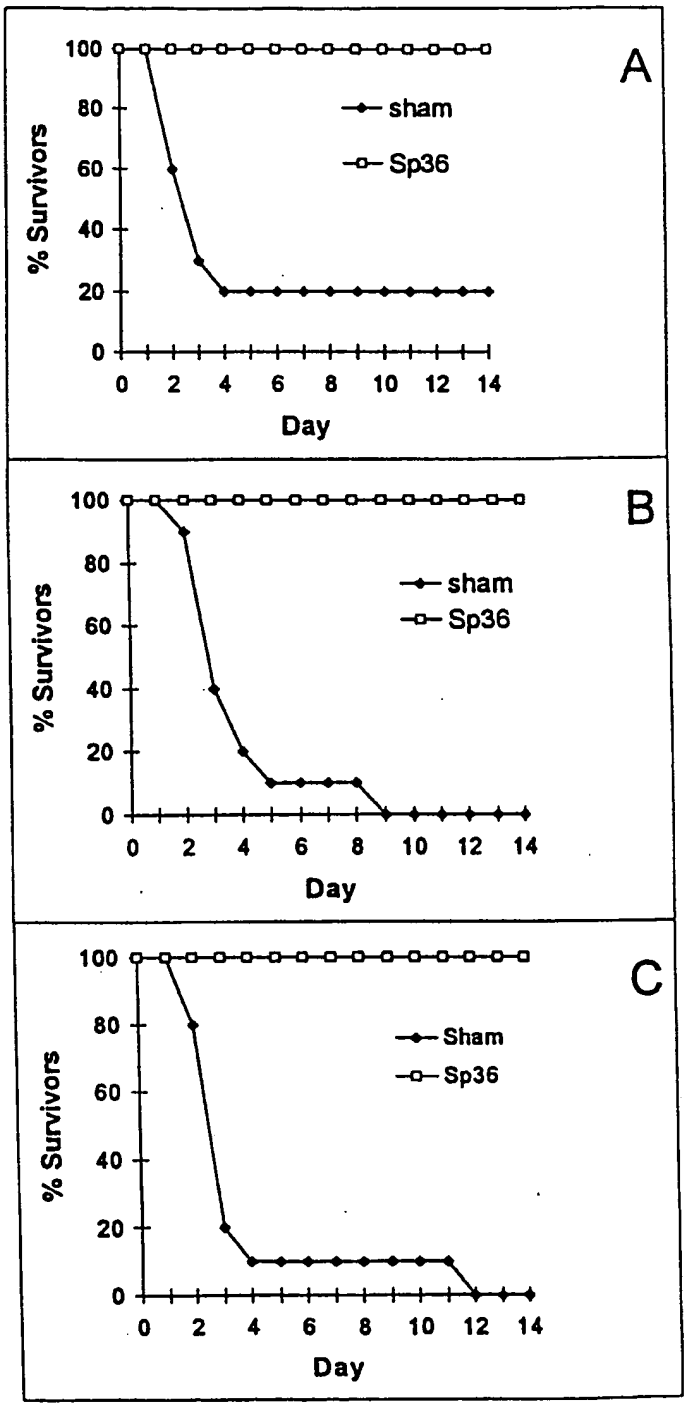
administering the vaccine of claim 1.

3. A vaccine composition comprising:

(a) at least one antibody against a member selected from the group consistng of (i) a polypeptide comprising a polypeptide sequence that

is at least 90% identical to amino acids 1-819 of SEQ ID NO:4; (ii) a polypeptide comprising a polypeptide sequence that is at least 90% identical to amino acids 1-460 of SEQ ID NO:6; (iii) a fragment of the polypeptide of (i) that includes at least one of histidine triad residue or coiled-coil region; (iv) a fragment of the polypeptide of (ii) that includes at least one of a histidine triad residue or a coiled-coil region; (v) a fragment of a polypeptide that is at least 90% identical to the polypeptide sequence comprising amino acids 1-800 of SEQ ID NO:8, wherein said fragment includes at least one of a histidine triad residue or coiled-coil region wherein said fragment includes at least 80 amino acids and no more than 680 amino acids and (vi) a fragment of a polypeptide that is at least 90% identical to the polypeptide sequence comprising amino acids 1-800 of SEQ ID NO:10, wherein said fragment includes at least one of a histidine triad residue or coiled-coil region wherein said fragment includes at least 80 amino acids and no more than 680 amino acids.

Figure 1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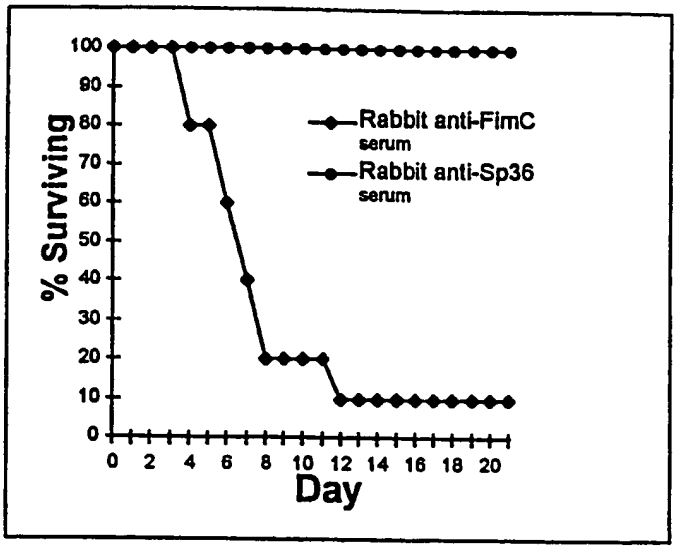
100
80
60
40
20
0
% Survivors
sham
Sp36
0 2 4 6 8 10 12 14
Day
B
100
80
60
40
20
0
% Survivors
sham
Sp36
0 2 4 6 8 10 12 14
Day
C
100
80
60
40
20
0
% Survivors
Sham
Sp36
0 2 4 6 8 10 12 14
Day

Figure 2

A. Strain SJ2 (serotype 6B)



B. Strain EF6796 (serotype 6A)

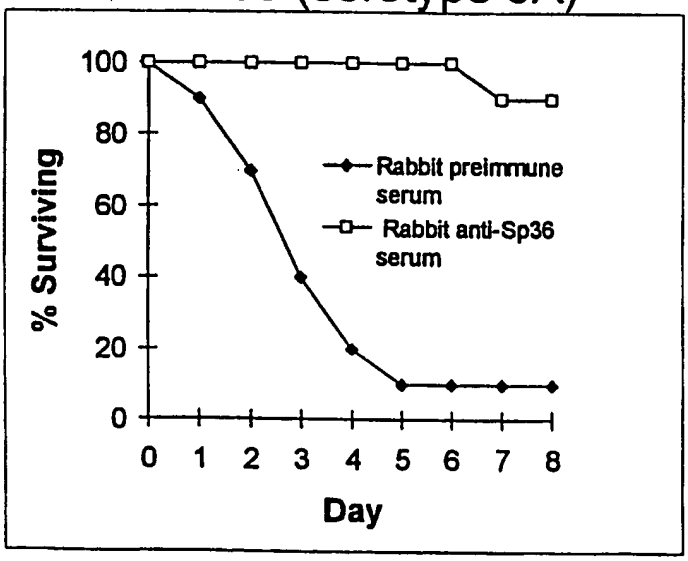


FIG. 3A

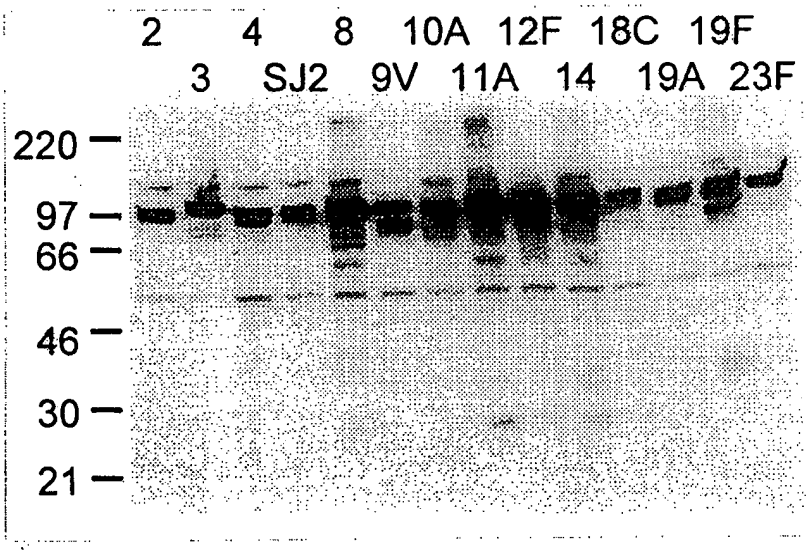

2
4
8
10A
12F
18C
19F
3
SJ2
9V
11A
14
19A
23F
220
97
66
46
30
21

FIG. 3B

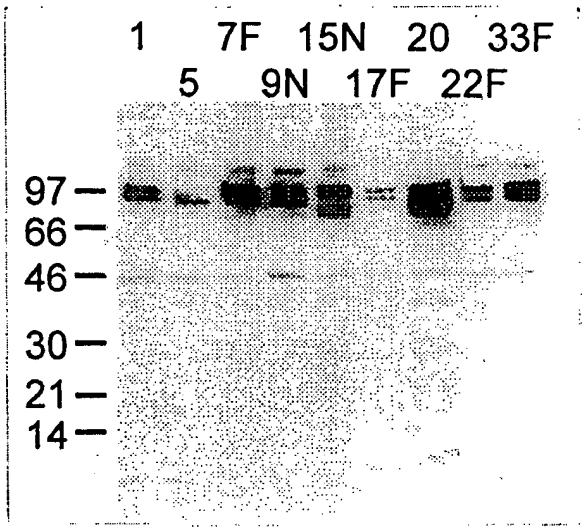

1
7F
15N
20
33F
5
9N
17F
22F
97
66
46
30
21
14

FIG. 4

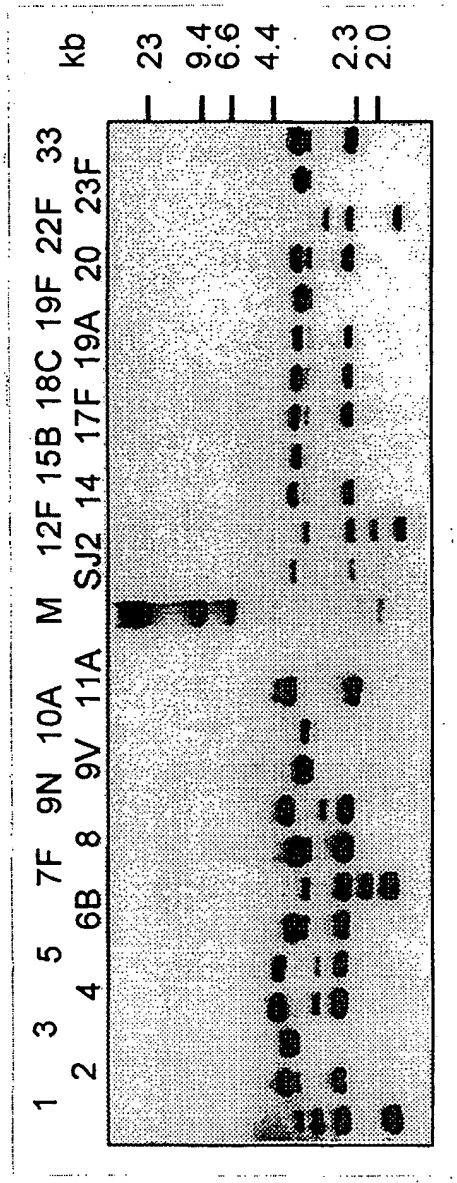
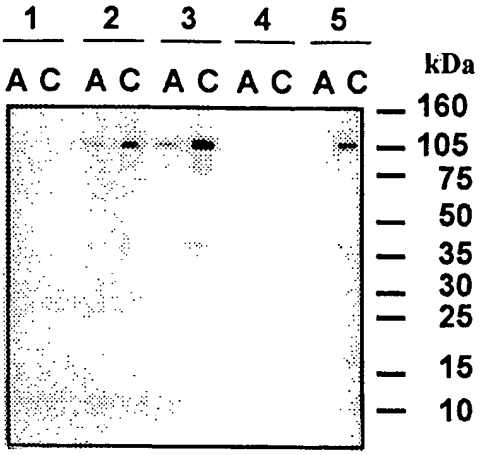
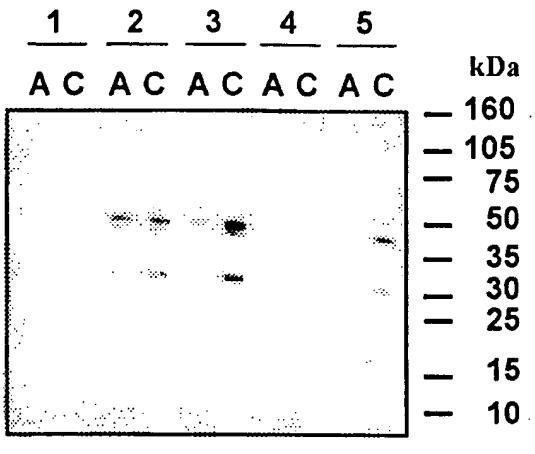

kb
23
9.4
6.6
4.4
2.3
2.0
1
2
3
4
5
6B
7F
8
9N
9V
10A
11A
M
SJ2
12F
14
15B
17F
18C
19A
19F
20
22F
23F
33

FIG. 5A



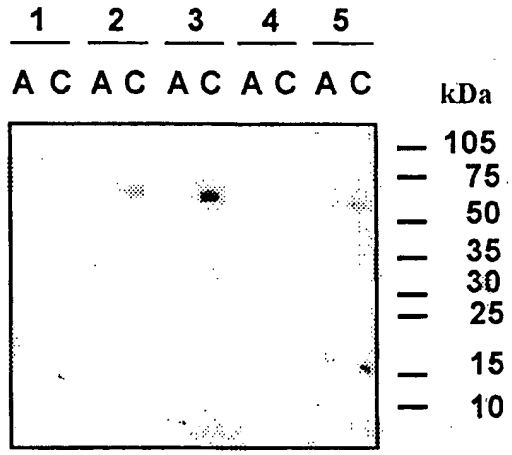
PhtA

FIG. 5B



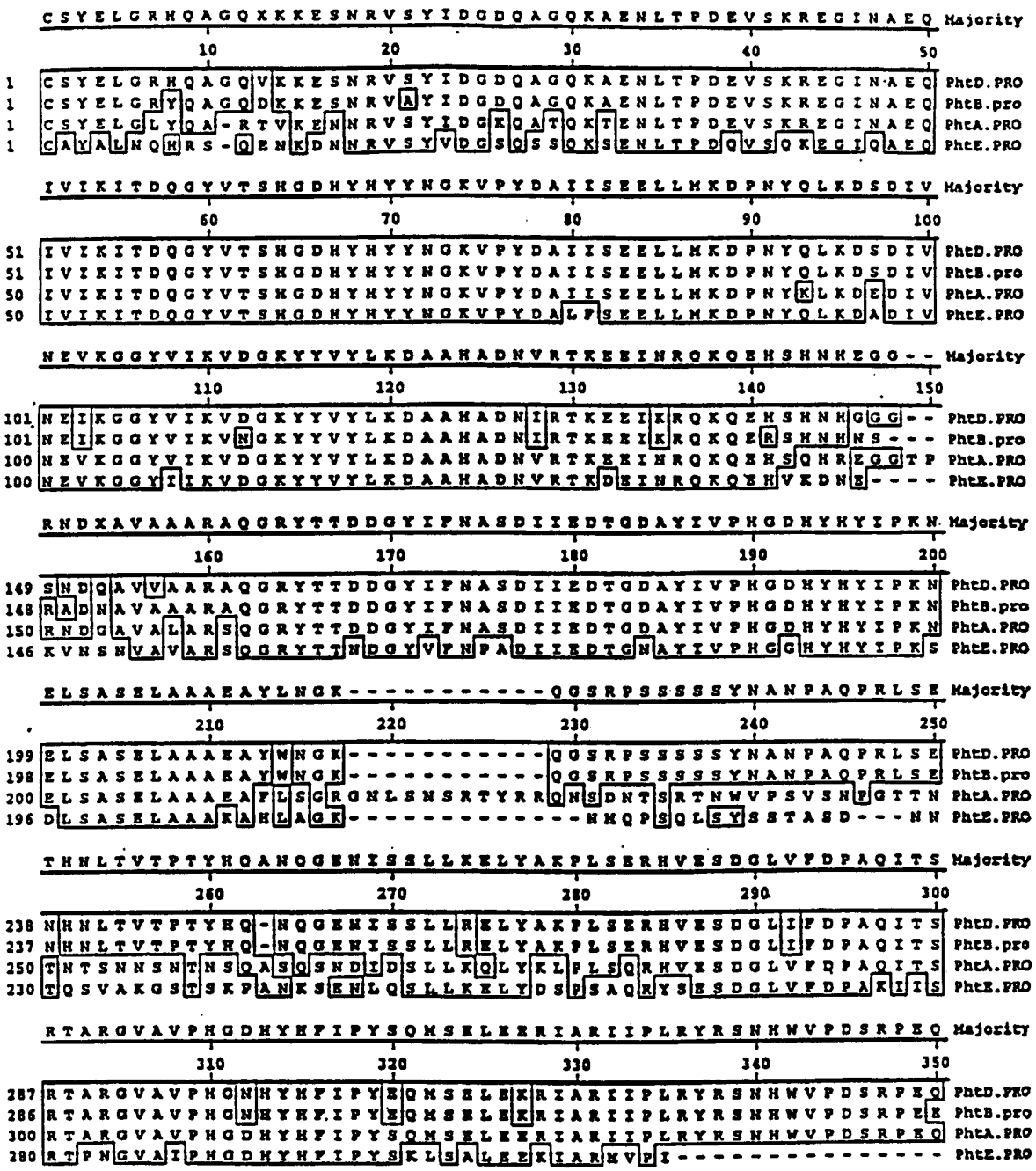
PhtA N-terminal

FIG. 5C



PhtA C-terminal

Figure 6(a)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1 C S Y E L G R Y Q A G Q D K K E S N R V A Y I D G D Q A G Q K A E N L T P D E V S K R E G I N A E Q PhtB.pro
1 C S Y E L G L Y Q A - R T V K E N N R V S Y I D G K Q A T Q K T E N L T P D E V S K R E G I N A E Q PhtA.PRO
1 C A Y A L N Q H R S - Q E N K D N N R V S Y V D G S Q S S Q K S E N L T P D Q V S Q K E G I Q A E Q PhtE.PRO
I V I K I T D Q G Y V T S H G D H Y H Y Y N G K V P Y D A I I S E E L L M K D P N Y Q L K D S D I V Majority
60 70 80 90 100
51 I V I K I T D Q G Y V T S H G D H Y H Y Y N G K V P Y D A I I S E E L L M K D P N Y Q L K D S D I V PhtD.PRO
51 I V I K I T D Q G Y V T S H G D H Y H Y Y N G K V P Y D A I I S E E L L M K D P N Y Q L K D S D I V PhtB.pro
50 I V I K I T D Q G Y V T S H G D H Y H Y Y N G K V P Y D A I I S E E L L M K D P N Y K L K D E D I V PhtA.PRO
50 I V I K I T D Q G Y V T S H G D H Y H Y Y N G K V P Y D A L F S E E L L M K D P N Y Q L K D A D I V PhtE.PRO
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110 120 130 140 150
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100 N E V K G G Y V I K V D G K Y Y V Y L K D A A H A D N V R T K E E I N R Q K Q E H S Q H R E G G T P PhtA.PRO
100 N E V K G G Y I I K V D G K Y Y V Y L K D A A H A D N V R T K D E I N R Q K Q E H V K D N E - - - - PhtE.PRO
R N D X A V A A A R A Q G R Y T T D D G Y I F N A S D I I E D T G D A Y I V P H G D H Y H Y I P K N Majority
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150 R N D G A V A L A R S Q G R Y T T D D G Y I F N A S D I I E D T G D A Y I V P H G D H Y H Y I P K N PhtA.PRO
146 K V N S N V A V A R S Q G R Y T T N D G Y V F N P A D I I E D T G N A Y I V P H G G H Y H Y I P K S PhtE.PRO
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210 220 230 240 250
199 E L S A S E L A A A E A Y W N G K - - - - - - - - - - - - Q G S R P S S S S S Y N A N P A Q P R L S E PhtD.PRO
198 E L S A S E L A A A E A Y W N G K - - - - - - - - - - - - Q G S R P S S S S S Y N A N P A Q P R L S E PhtB.pro
200 E L S A S E L A A A E A F L S G R G N L S N S R T Y R R Q N S D N T S R T N W V P S V S N P G T T N PhtA.PRO
196 D L S A S E L A A A K A H L A G K - - - - - - - - - - - - - - H M Q P S Q L S Y S S T A S D - - - N N PhtE.PRO
T H N L T V T P T Y H Q A N Q G E N I S S L L K E L Y A K P L S E R H V E S D G L V F D P A Q I T S Majority
260 270 280 290 300
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Figure 6(b)

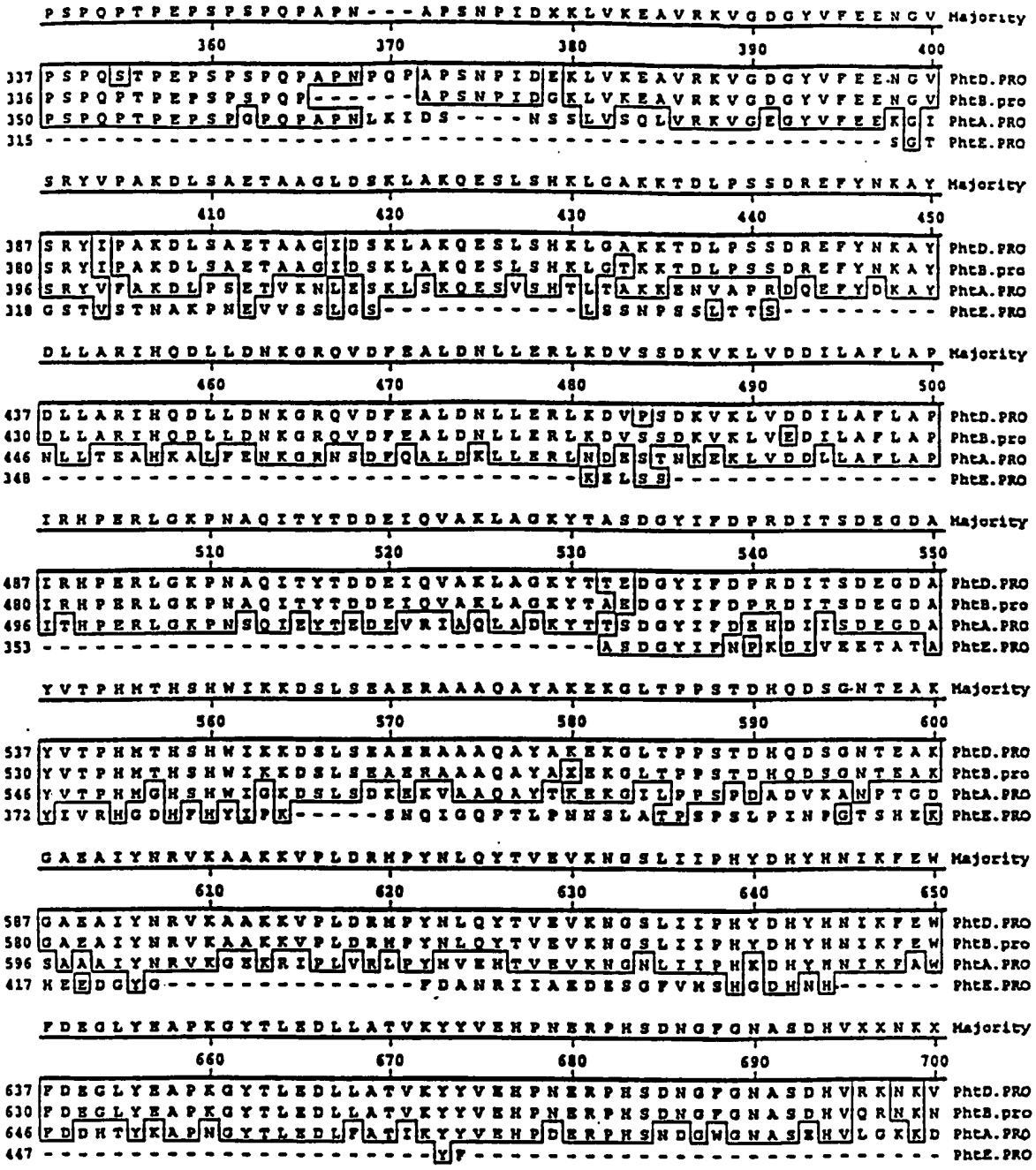
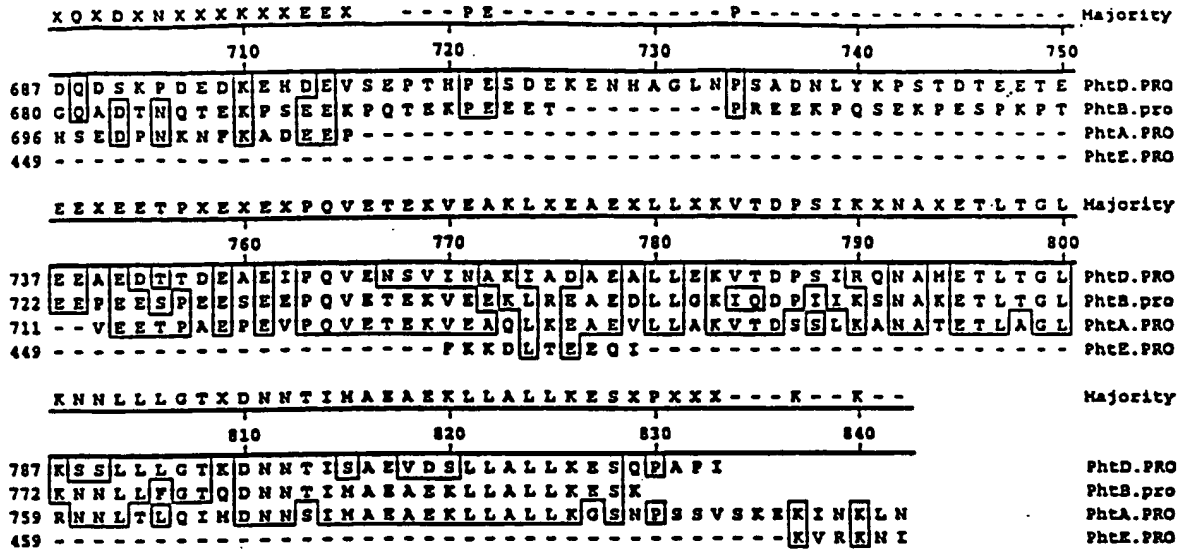


Figure 6(c)



Decoration 'Decoration #2': Box residues that match the Consensus exactly.

Figure 7(a)

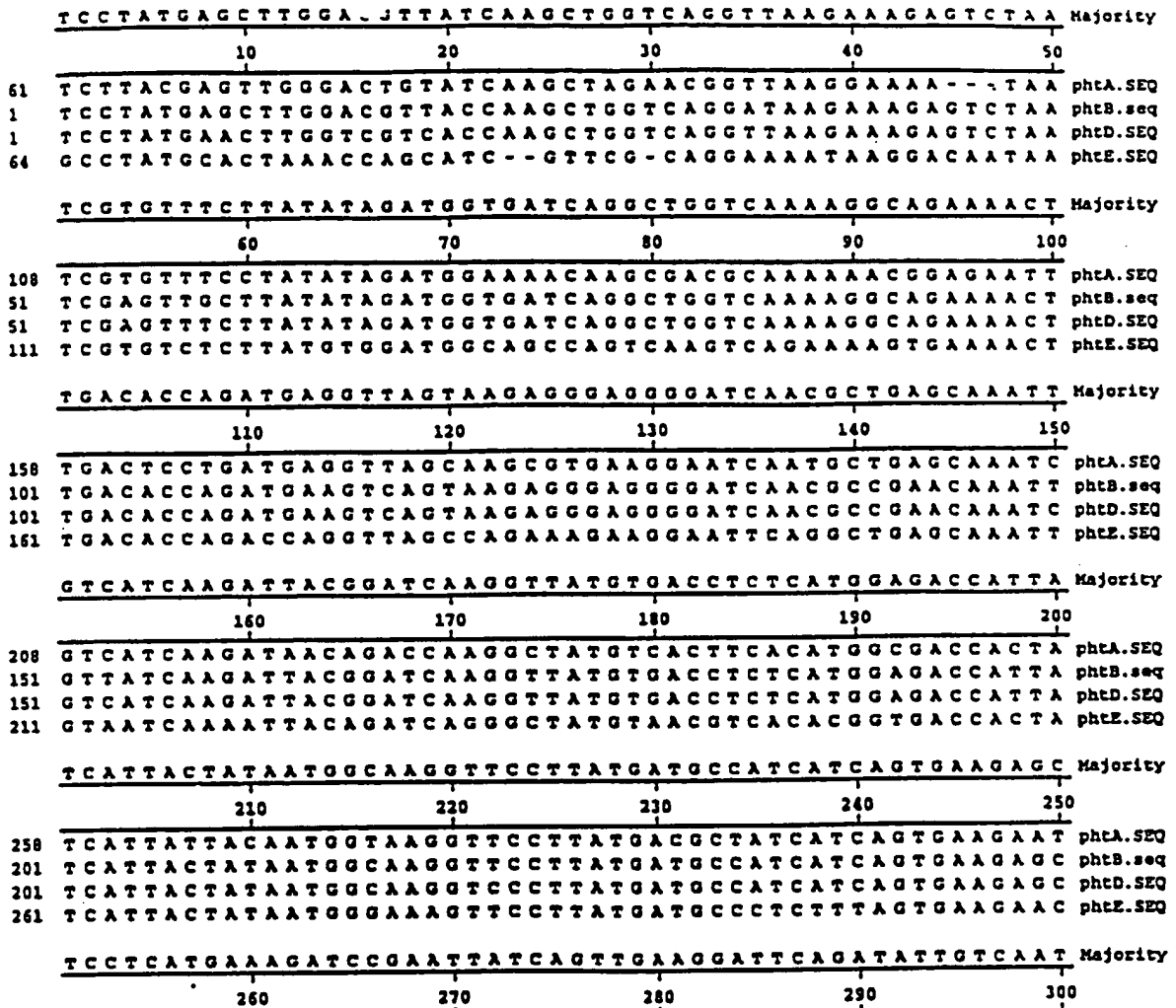

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Figure 7(b)

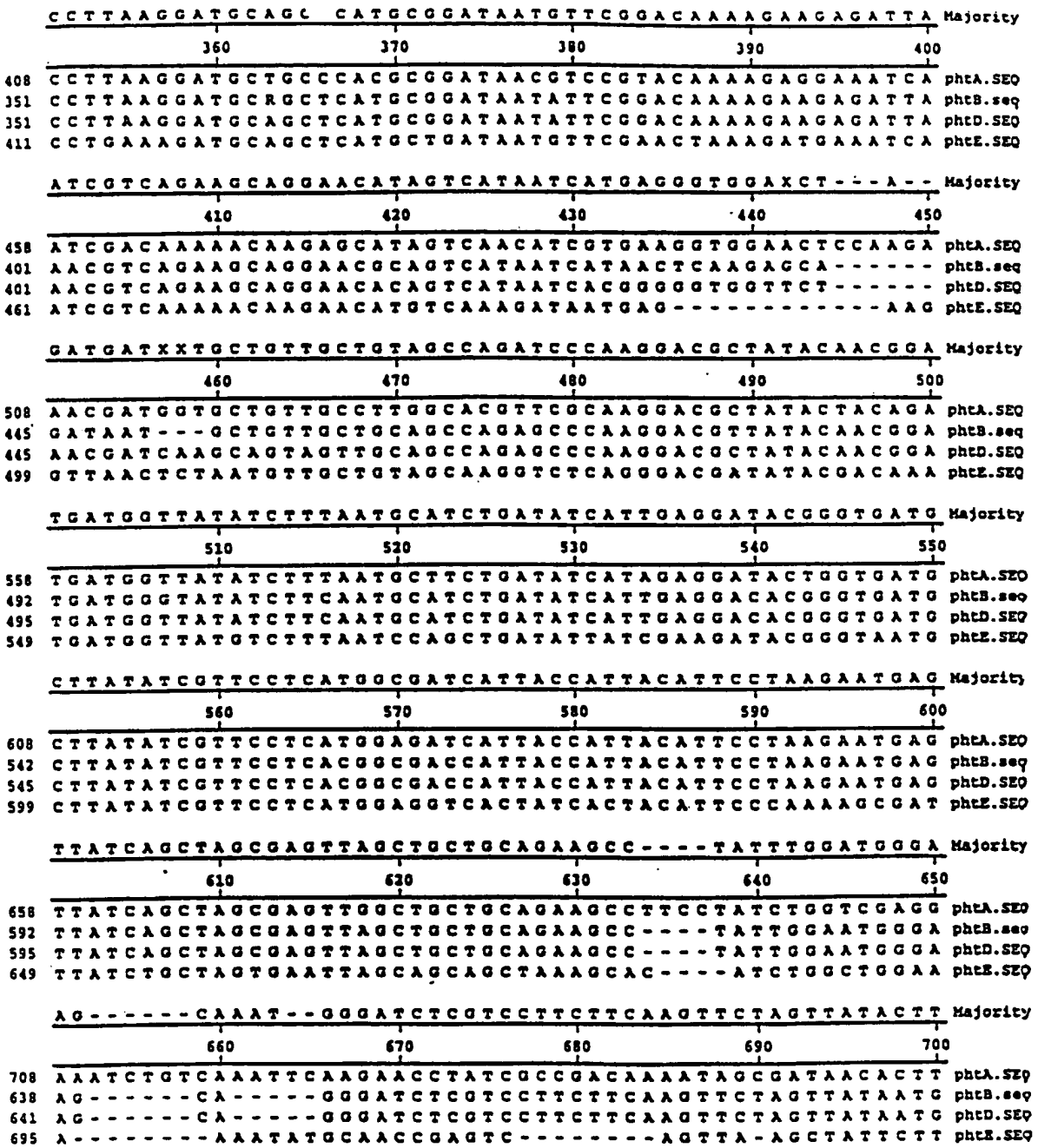
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Figure 7(c)

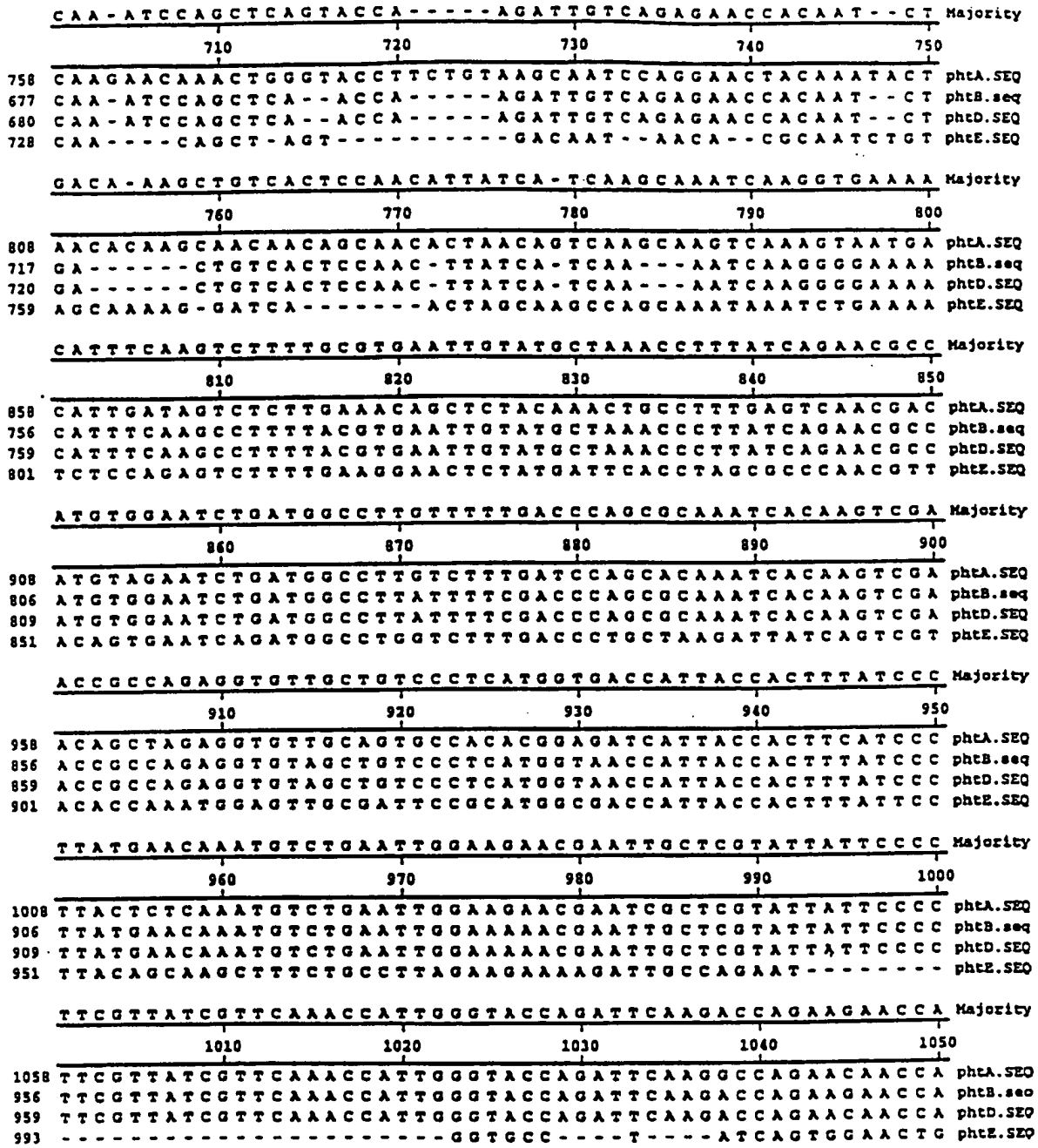
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Figure 7(d)

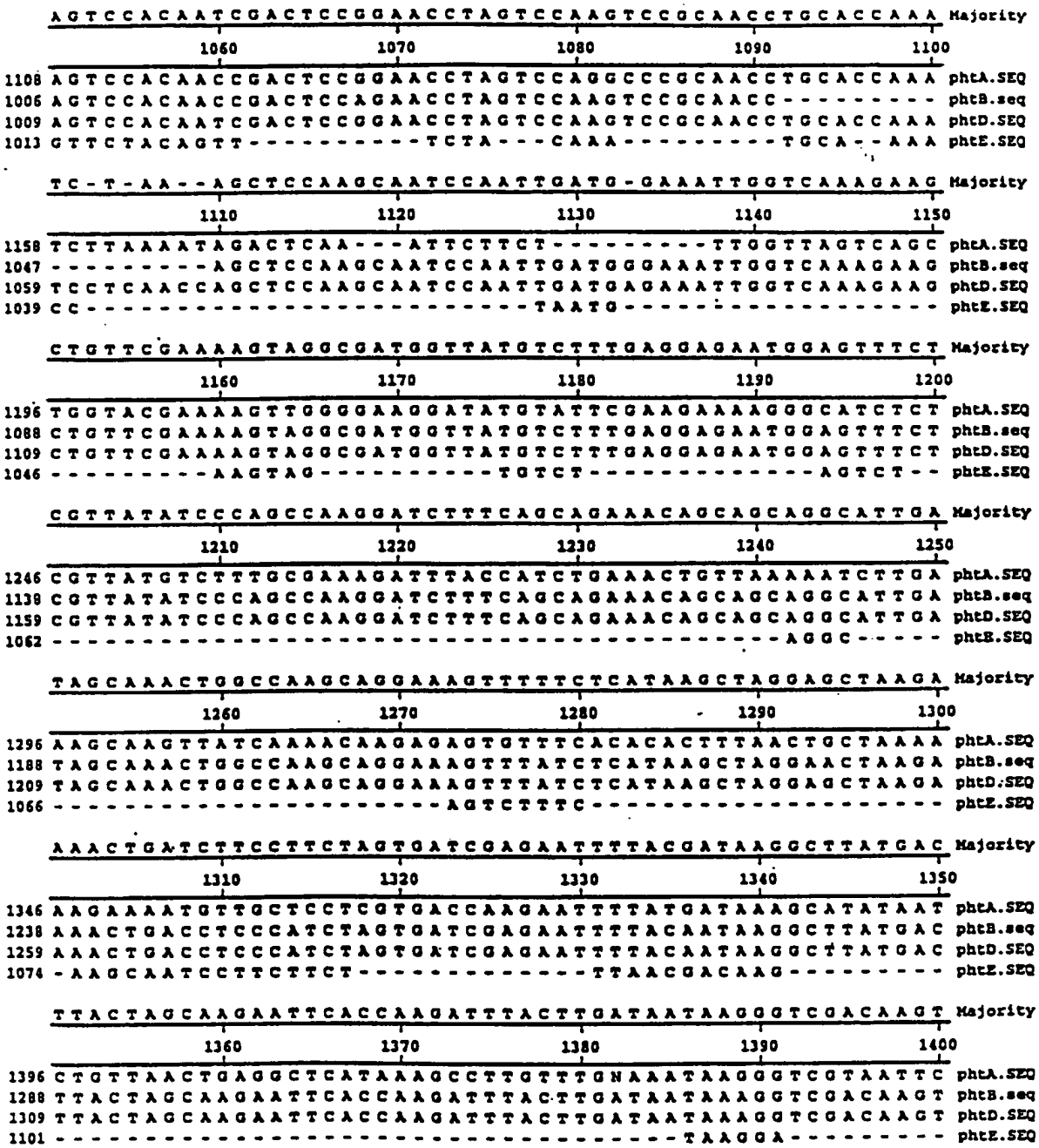
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Figure 7(e)

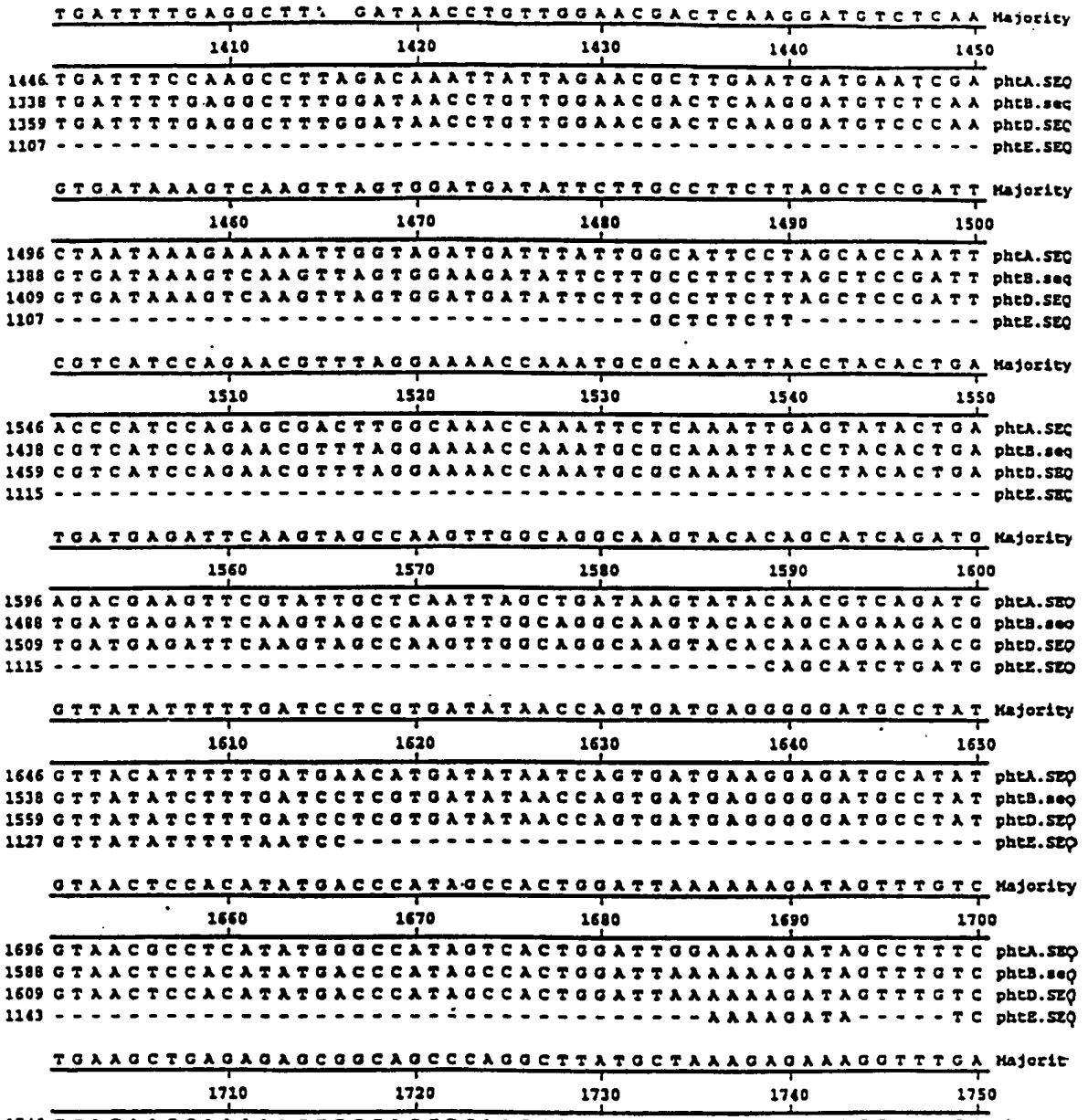
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Figure 7(f)

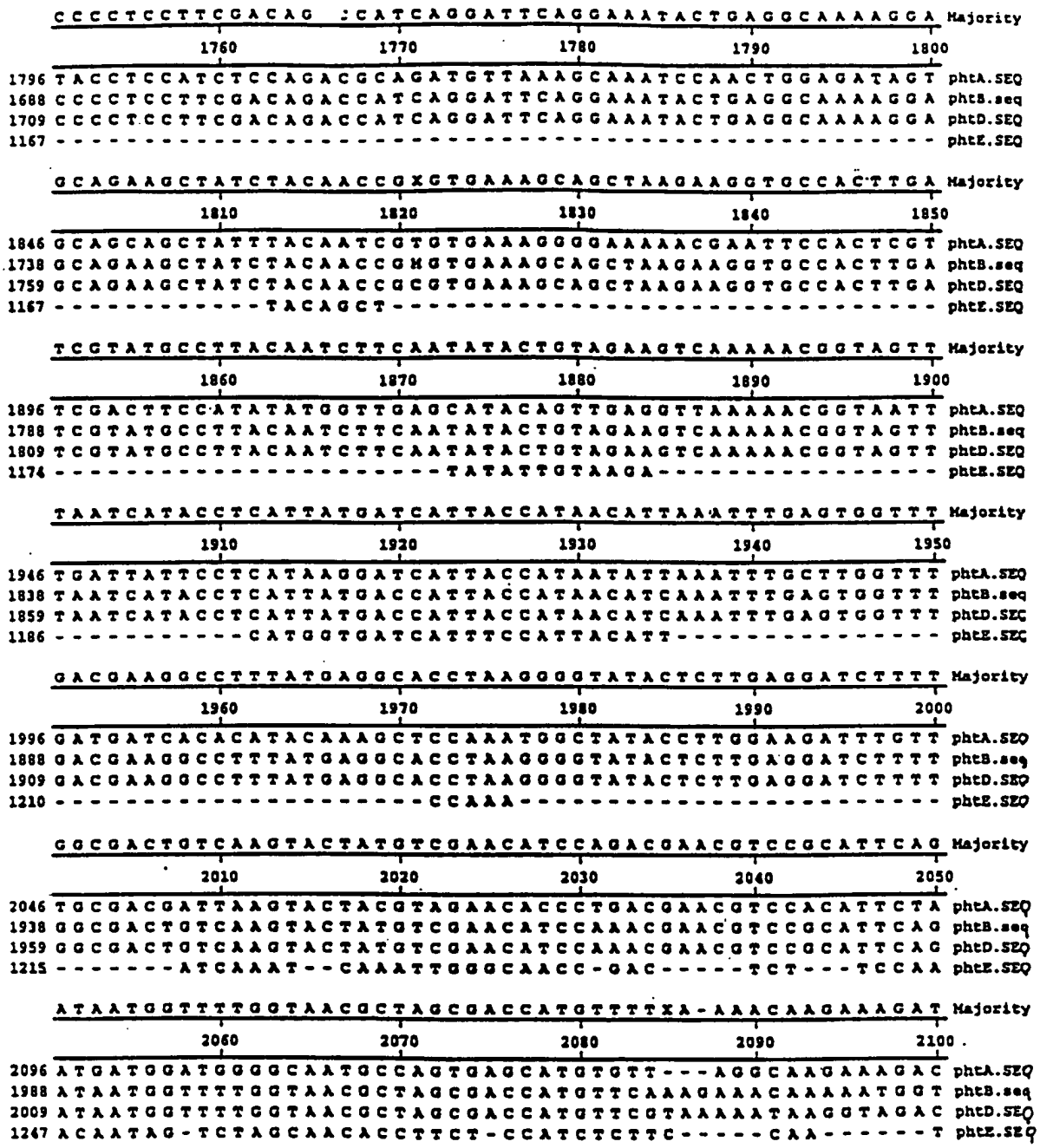
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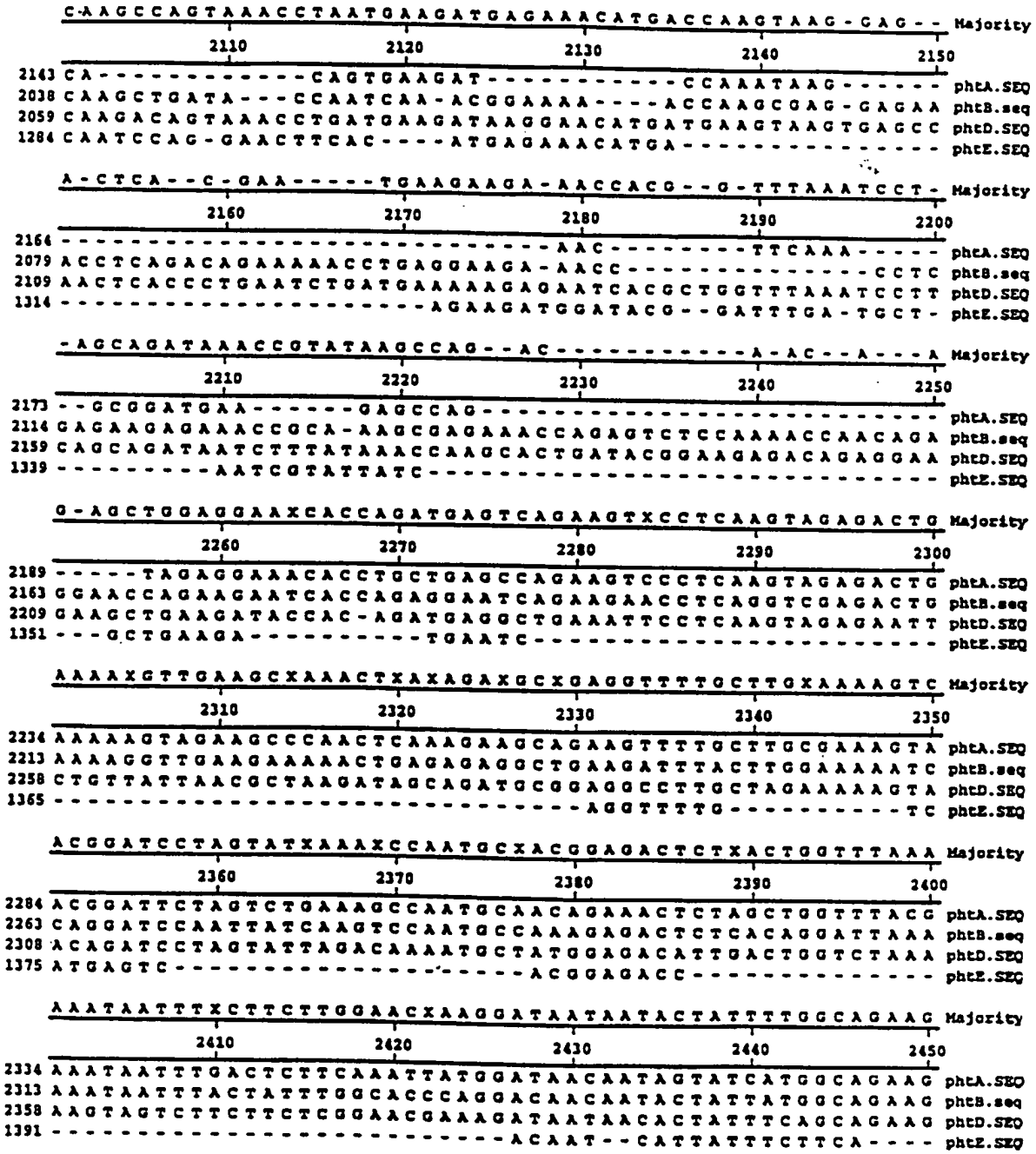
Figure 7(g)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Majority
2160 2170 2180 2190 2200
2164 phtA.SEQ
2079 phtB.seq
2109 phtD.SEQ
1314 phtE.SEQ
Majority
2210 2220 2230 2240 2250
2173 phtA.SEQ
2116 phtB.seq
2159 phtD.SEQ
1339 phtE.SEQ
Majority
2260 2270 2280 2290 2300
2189 phtA.SEQ
2163 phtB.seq
2209 phtD.SEQ
1351 phtE.SEQ
Majority
2310 2320 2330 2340 2350
2234 phtA.SEQ
2213 phtB.seq
2258 phtD.SEQ
1365 phtE.SEQ
Majority
2360 2370 2380 2390 2400
2284 phtA.SEQ
2263 phtB.seq
2308 phtD.SEQ
1375 phtE.SEQ
Majority
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Figure 7(h)

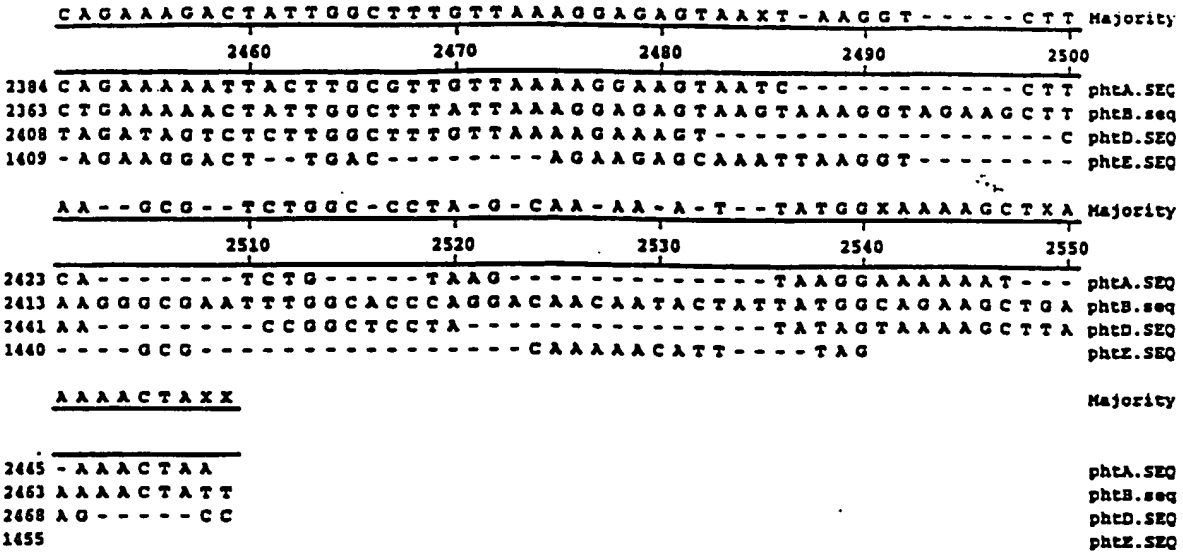
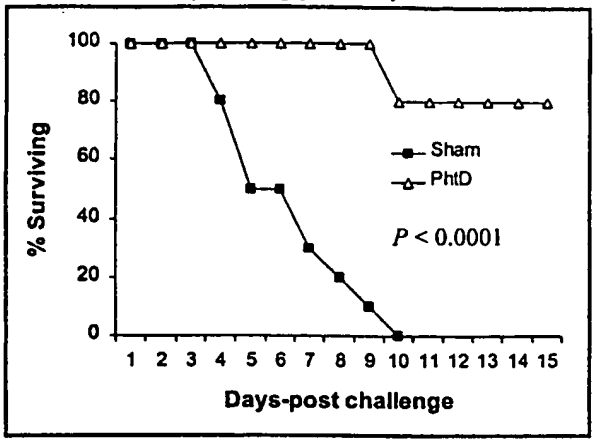
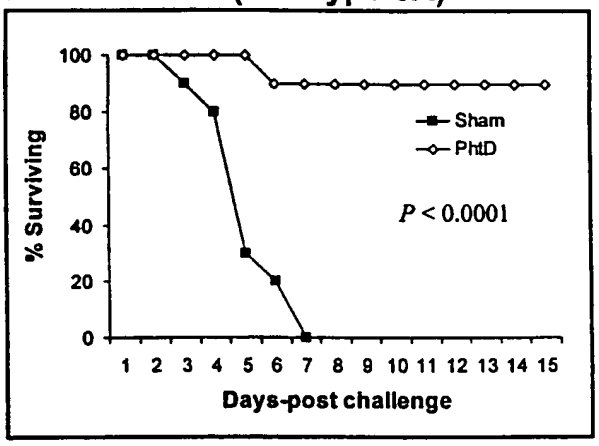

CAGAAAGACTATTGGCTTTGTTAAAGGAGAGTAAXT-AAGGT-----CTT Majority
2460 2470 2480 2490 2500
2384 CAGAAAAATTACTTGCGTTGTTAAAAGGAAGTAATC----------CTT phtA.SEQ
2363 CTGAAAAACTATTGGCTTTATTAAAGGAGAGTAAGTAAAGGTAGAAGCTT phtB.seq
2408 TAGATAGTCTCTTGGCTTTGTTAAAAGAAAGT----------------C phtD.SEQ
1409 -AGAAGGACT--TGAC---------AGAAGAGCAAATTAAGGT-------- phtE.SEQ
AA--GCG--TCTGGC-CCTA-G-CAA-AA-A-T--TATGGXAAAAAGCTXA Majority
2510 2520 2530 2540 2550
2423 CA-------TCTG-----TAAG----------TAAGGAAAAAAT--- phtA.SEQ
2413 AAGGGCGAATTTGGCACCCAGGACAACAATACTATTATGGCAGAAGCTGA phtB.seq
2441 AA--------CCGGCTCCTA----------TATAGTAAAAGCTTA phtD.SEQ
1440 ----GCG----------CAAAAACATT----TAG phtE.SEQ
AAAACTAXX Majority
2465 -AAACTAA phtA.SEQ
2463 AAAACTATT phtB.seq
2468 AG-----CC phtD.SEQ
1455 phtE.SEQ

Figure 8

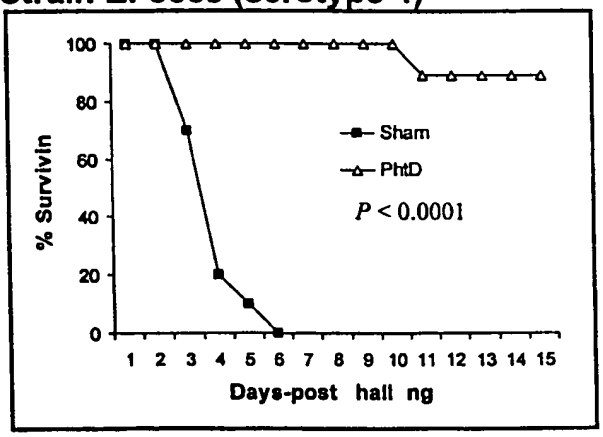
A. Strain SJ2 (serotype 6B)



B. Strain EF6796 (serotype 6A)



C. Strain EF5668 (serotype 4)



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Koenig, Scott
Adamou, John E.

<120> Streptococcus Pneumoniae and Immunogenic Fragments for Vaccines

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Lys Lys Glu Ser Asn Arg Val Ser Tyr Ile Asp Gly Asp Gln Ala Gly
35 40 45

Gln Lys Ala Glu Asn Leu Thr Pro Asp Glu Val Ser Lys Arg Glu Gly
50 55 60

Ile Asn Ala Glu Gln Ile Val Ile Lys Ile Thr Asp Gln Gly Tyr Val
65 70 75 80

Thr Ser His Gly Asp His Tyr His Tyr Tyr Asn Gly Lys Val Pro Tyr
85 90 95

Asp Ala Ile Ile Ser Glu Glu Leu Leu Met Lys Asp Pro Asn Tyr Gln
100 105 110

Leu Lys Asp Ser Asp Ile Val Asn Glu Ile Lys Gly Gly Tyr Val Ile
115 120 125

Lys Val Asp Gly Lys Tyr Tyr Val Tyr Leu Lys Asp Ala Ala His Ala
130 135 140

Asp Asn Ile Arg Thr Lys Glu Glu Ile Lys Arg Gln Lys Gln Glu His
145 150 155 160

Ser His Asn His Gly Gly Gly Ser Asn Asp Gln Ala Val Val Ala Ala
165 170 175

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Arg Ala Gln Gly Arg Tyr Thr Thr Asp Asp Gly Tyr Ile Phe Asn Ala
            180                 185                 190

Ser Asp Ile Ile Glu Asp Thr Gly Asp Ala Tyr Ile Val Pro His Gly
        195                 200                 205

Asp His Tyr His Tyr Ile Pro Lys Asn Glu Leu Ser Ala Ser Glu Leu
    210                 215                 220

Ala Ala Ala Glu Ala Tyr Trp Asn Gly Lys Gln Gly Ser Arg Pro Ser
225                 230                 235                 240

Ser Ser Ser Ser Tyr Asn Ala Asn Pro Ala Gln Pro Arg Leu Ser Glu
                245                 250                 255

Asn His Asn Leu Thr Val Thr Pro Thr Tyr His Gln Asn Gln Gly Glu
            260                 265                 270

Asn Ile Ser Ser Leu Leu Arg Glu Leu Tyr Ala Lys Pro Leu Ser Glu
        275                 280                 285

Arg His Val Glu Ser Asp Gly Leu Ile Phe Asp Pro Ala Gln Ile Thr
    290                 295                 300

Ser Arg Thr Ala Arg Gly Val Ala Val Pro His Gly Asn His Tyr His
305                 310                 315                 320

Phe Ile Pro Tyr Glu Gln Met Ser Glu Leu Glu Lys Arg Ile Ala Arg
                325                 330                 335

Ile Ile Pro Leu Arg Tyr Arg Ser Asn His Trp Val Pro Asp Ser Arg
            340                 345                 350

Pro Glu Gln Pro Ser Pro Gln Ser Thr Pro Glu Pro Ser Pro Ser Pro
        355                 360                 365

Gln Pro Ala Pro Asn Pro Gln Pro Ala Pro Ser Asn Pro Ile Asp Glu
    370                 375                 380

Lys Leu Val Lys Glu Ala Val Arg Lys Val Gly Asp Gly Tyr Val Phe
385                 390                 395                 400

Glu Glu Asn Gly Val Ser Arg Tyr Ile Pro Ala Lys Asp Leu Ser Ala
                405                 410                 415

Glu Thr Ala Ala Gly Ile Asp Ser Lys Leu Ala Lys Gln Glu Ser Leu
            420                 425                 430
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Ser His Lys Leu Gly Ala Lys Lys Thr Asp Leu Pro Ser Ser Asp Arg
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Glu Phe Tyr Asn Lys Ala Tyr Asp Leu Leu Ala Arg Ile His Gln Asp
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Leu Leu Asp Asn Lys Gly Arg Gln Val Asp Phe Glu Ala Leu Asp Asn
465 470 475 480

Leu Leu Glu Arg Leu Lys Asp Val Pro Ser Asp Lys Val Lys Leu Val
485 490 495

Asp Asp Ile Leu Ala Phe Leu Ala Pro Ile Arg His Pro Glu Arg Leu
500 505 510

Gly Lys Pro Asn Ala Gln Ile Thr Tyr Thr Asp Asp Glu Ile Gln Val
515 520 525

Ala Lys Leu Ala Gly Lys Tyr Thr Thr Glu Asp Gly Tyr Ile Phe Asp
530 535 540

Pro Arg Asp Ile Thr Ser Asp Glu Gly Asp Ala Tyr Val Thr Pro His
545 550 555 560

Met Thr His Ser His Trp Ile Lys Lys Asp Ser Leu Ser Glu Ala Glu
565 570 575

Arg Ala Ala Ala Gln Ala Tyr Ala Lys Glu Lys Gly Leu Thr Pro Pro
580 585 590

Ser Thr Asp His Gln Asp Ser Gly Asn Thr Glu Ala Lys Gly Ala Glu
595 600 605

Ala Ile Tyr Asn Arg Val Lys Ala Ala Lys Lys Val Pro Leu Asp Arg
610 615 620

Met Pro Tyr Asn Leu Gln Tyr Thr Val Glu Val Lys Asn Gly Ser Leu
625 630 635 640

Ile Ile Pro His Tyr Asp His Tyr His Asn Ile Lys Phe Glu Trp Phe
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Asp Glu Gly Leu Tyr Glu Ala Pro Lys Gly Tyr Thr Leu Glu Asp Leu
660 665 670

Leu Ala Thr Val Lys Tyr Tyr Val Glu His Pro Asn Glu Arg Pro His
675 680 685

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Ser Asp Asn Gly Phe Gly Asn Ala Ser Asp His Val Arg Lys Asn Lys
    690                 695                 700

Val Asp Gln Asp Ser Lys Pro Asp Glu Asp Lys Glu His Asp Glu Val
705                 710                 715                 720

Ser Glu Pro Thr His Pro Glu Ser Asp Glu Lys Glu Asn His Ala Gly
                725                 730                 735

Leu Asn Pro Ser Ala Asp Asn Leu Tyr Lys Pro Ser Thr Asp Thr Glu
            740                 745                 750

Glu Thr Glu Glu Glu Ala Glu Asp Thr Thr Asp Glu Ala Glu Ile Pro
        755                 760                 765

Gln Val Glu Asn Ser Val Ile Asn Ala Lys Ile Ala Asp Ala Glu Ala
    770                 775                 780

Leu Leu Glu Lys Val Thr Asp Pro Ser Ile Arg Gln Asn Ala Met Glu
785                 790                 795                 800

Thr Leu Thr Gly Leu Lys Ser Ser Leu Leu Leu Gly Thr Lys Asp Asn
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Asn Thr Ile Ser Ala Glu Val Asp Ser Leu Leu Ala Leu Leu Lys Glu
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Ser Gln Pro Ala Pro Ile
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aagagggagg ggatcaacgc cgaacaaatc gtcatcaaga ttacggatca aggttatgtg 240
acctctcatg gagaccatta tcattactat aatggcaagg tcccttatga tgccatcatc 300
agtgaagagc tcctcatgaa agatccgaat tatcagttga aggattcaga cattgtcaat 360
gaaatcaagg gtggttatgt tatcaaggta gatggaaaat actatgttta ccttaaggat 420
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cgctatacaa cggatgatgg ttatatcttc aatgcatctg atatcattga ggacacgggt 600
gatgcttata tcgttcctca cggcgaccat taccattaca ttcctaagaa tgagttatca 660
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actgtcactc caacttatca tcaaaatcaa ggggaaaaca tttcaagcct tttacgtgaa 840
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35 40 45

Gln Lys Ser Glu Asn Leu Thr Pro Asp Gln Val Ser Gln Lys Glu Gly
50 55 60

Ile Gln Ala Glu Gln Ile Val Ile Lys Ile Thr Asp Gln Gly Tyr Val
65 70 75 80

Thr Ser His Gly Asp His Tyr His Tyr Tyr Asn Gly Lys Val Pro Tyr
85 90 95

Asp Ala Leu Phe Ser Glu Glu Leu Leu Met Lys Asp Pro Asn Tyr Gln
100 105 110

Leu Lys Asp Ala Asp Ile Val Asn Glu Val Lys Gly Gly Tyr Ile Ile
115 120 125

Lys Val Asp Gly Lys Tyr Tyr Val Tyr Leu Lys Asp Ala Ala His Ala
130 135 140

Asp Asn Val Arg Thr Lys Asp Glu Ile Asn Arg Gln Lys Gln Glu His
145 150 155 160

Val Lys Asp Asn Glu Lys Val Asn Ser Asn Val Ala Val Ala Arg Ser
165 170 175

Gln Gly Arg Tyr Thr Thr Asn Asp Gly Tyr Val Phe Asn Pro Ala Asp
180 185 190

Ile Ile Glu Asp Thr Gly Asn Ala Tyr Ile Val Pro His Gly Gly His
195 200 205

Tyr His Tyr Ile Pro Lys Ser Asp Leu Ser Ala Ser Glu Leu Ala Ala
210 215 220

Ala Lys Ala His Leu Ala Gly Lys Asn Met Gln Pro Ser Gln Leu Ser
225 230 235 240

Tyr Ser Ser Thr Ala Ser Asp Asn Asn Thr Gln Ser Val Ala Lys Gly
245 250 255

Ser Thr Ser Lys Pro Ala Asn Lys Ser Glu Asn Leu Gln Ser Leu Leu
260 265 270

Lys Glu Leu Tyr Asp Ser Pro Ser Ala Gln Arg Tyr Ser Glu Ser Asp
275 280 285

Gly Leu Val Phe Asp Pro Ala Lys Ile Ile Ser Arg Thr Pro Asn Gly
290 295 300

Val Ala Ile Pro His Gly Asp His Tyr His Phe Ile Pro Tyr Ser Lys
305 310 315 320

Leu Ser Ala Leu Glu Glu Lys Ile Ala Arg Met Val Pro Ile Ser Gly
325 330 335

Thr Gly Ser Thr Val Ser Thr Asn Ala Lys Pro Asn Glu Val Val Ser
340 345 350

Ser Leu Gly Ser Leu Ser Ser Asn Pro Ser Ser Leu Thr Thr Ser Lys
355 360 365

Glu Leu Ser Ser Ala Ser Asp Gly Tyr Ile Phe Asn Pro Lys Asp Ile
370 375 380

Val Glu Glu Thr Ala Thr Ala Tyr Ile Val Arg His Gly Asp His Phe
385 390 395 400

His Tyr Ile Pro Lys Ser Asn Gln Ile Gly Gln Pro Thr Leu Pro Asn
405 410 415

Asn Ser Leu Ala Thr Pro Ser Pro Ser Leu Pro Ile Asn Pro Gly Thr
420 425 430

Ser His Glu Lys His Glu Glu Asp Gly Tyr Gly Phe Asp Ala Asn Arg
435 440 445

Ile Ile Ala Glu Asp Glu Ser Gly Phe Val Met Ser His Gly Asp His
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Arg Lys Asn Ile

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35 40 45

Lys Thr Glu Asn Leu Thr Pro Asp Glu Val Ser Lys Arg Glu Gly Ile
50 55 60

Asn Ala Glu Gln Ile Val Ile Lys Ile Thr Asp Gln Gly Tyr Val Thr
65 70 75 80

Ser His Gly Asp His Tyr His Tyr Tyr Asn Gly Lys Val Pro Tyr Asp
85 90 95

Ala Ile Ile Ser Glu Glu Leu Leu Met Lys Asp Pro Asn Tyr Lys Leu
100 105 110

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Val Asp Gly Lys Tyr Tyr Val Tyr Leu Lys Asp Ala Ala His Ala Asp
    130                 135                 140

Asn Val Arg Thr Lys Glu Glu Ile Asn Arg Gln Lys Gln Glu His Ser
145                 150                 155                 160

Gln His Arg Glu Gly Gly Thr Pro Arg Asn Asp Gly Ala Val Ala Leu
                165                 170                 175

Ala Arg Ser Gln Gly Arg Tyr Thr Thr Asp Asp Gly Tyr Ile Phe Asn
            180                 185                 190

Ala Ser Asp Ile Ile Glu Asp Thr Gly Asp Ala Tyr Ile Val Pro His
        195                 200                 205

Gly Asp His Tyr His Tyr Ile Pro Lys Asn Glu Leu Ser Ala Ser Glu
    210                 215                 220

Leu Ala Ala Ala Glu Ala Phe Leu Ser Gly Arg Gly Asn Leu Ser Asn
225                 230                 235                 240

Ser Arg Thr Tyr Arg Arg Gln Asn Ser Asp Asn Thr Ser Arg Thr Asn
                245                 250                 255

Trp Val Pro Ser Val Ser Asn Pro Gly Thr Thr Asn Thr Asn Thr Ser
            260                 265                 270

Asn Asn Ser Asn Thr Asn Ser Gln Ala Ser Gln Ser Asn Asp Ile Asp
        275                 280                 285

Ser Leu Leu Lys Gln Leu Tyr Lys Leu Pro Leu Ser Gln Arg His Val
    290                 295                 300

Glu Ser Asp Gly Leu Val Phe Asp Pro Ala Gln Ile Thr Ser Arg Thr
305                 310                 315                 320

Ala Arg Gly Val Ala Val Pro His Gly Asp His Tyr His Phe Ile Pro
                325                 330                 335

Tyr Ser Gln Met Ser Glu Leu Glu Glu Arg Ile Ala Arg Ile Ile Pro
            340                 345                 350

Leu Arg Tyr Arg Ser Asn His Trp Val Pro Asp Ser Arg Pro Glu Gln
        355                 360                 365
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Pro Asn Leu Lys Ile Asp Ser Asn Ser Ser Leu Val Ser Gln Leu Val
385                 390                 395                 400

Arg Lys Val Gly Glu Gly Tyr Val Phe Glu Glu Lys Gly Ile Ser Arg
                405                 410                 415

Tyr Val Phe Ala Lys Asp Leu Pro Ser Glu Thr Val Lys Asn Leu Glu
            420                 425                 430

Ser Lys Leu Ser Lys Gln Glu Ser Val Ser His Thr Leu Thr Ala Lys
        435                 440                 445

Lys Glu Asn Val Ala Pro Arg Asp Gln Glu Phe Tyr Asp Lys Ala Tyr
    450                 455                 460

Asn Leu Leu Thr Glu Ala His Lys Ala Leu Phe Glu Asn Lys Gly Arg
465                 470                 475                 480

Asn Ser Asp Phe Gln Ala Leu Asp Lys Leu Leu Glu Arg Leu Asn Asp
                485                 490                 495

Glu Ser Thr Asn Lys Glu Lys Leu Val Asp Asp Leu Leu Ala Phe Leu
            500                 505                 510

Ala Pro Ile Thr His Pro Glu Arg Leu Gly Lys Pro Asn Ser Gln Ile
        515                 520                 525

Glu Tyr Thr Glu Asp Glu Val Arg Ile Ala Gln Leu Ala Asp Lys Tyr
    530                 535                 540

Thr Thr Ser Asp Gly Tyr Ile Phe Asp Glu His Asp Ile Ile Ser Asp
545                 550                 555                 560

Glu Gly Asp Ala Tyr Val Thr Pro His Met Gly His Ser His Trp Ile
                565                 570                 575

Gly Lys Asp Ser Leu Ser Asp Lys Glu Lys Val Ala Ala Gln Ala Tyr
            580                 585                 590

Thr Lys Glu Lys Gly Ile Leu Pro Pro Ser Pro Asp Ala Asp Val Lys
        595                 600                 605

Ala Asn Pro Thr Gly Asp Ser Ala Ala Ala Ile Tyr Asn Arg Val Lys
    610                 615                 620
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Gly Glu Lys Arg Ile Pro Leu Val Arg Leu Pro Tyr Met Val Glu His
625 630 635 640

Thr Val Glu Val Lys Asn Gly Asn Leu Ile Ile Pro His Lys Asp His
645 650 655

Tyr His Asn Ile Lys Phe Ala Trp Phe Asp Asp His Thr Tyr Lys Ala
660 665 670

Pro Asn Gly Tyr Thr Leu Glu Asp Leu Phe Ala Thr Ile Lys Tyr Tyr
675 680 685

Val Glu His Pro Asp Glu Arg Pro His Ser Asn Asp Gly Trp Gly Asn
690 695 700

Ala Ser Glu His Val Leu Gly Lys Lys Asp His Ser Glu Asp Pro Asn
705 710 715 720

Lys Asn Phe Lys Ala Asp Glu Glu Pro Val Glu Glu Thr Pro Ala Glu
725 730 735

Pro Glu Val Pro Gln Val Glu Thr Glu Lys Val Glu Ala Gln Leu Lys
740 745 750

Glu Ala Glu Val Leu Leu Ala Lys Val Thr Asp Ser Ser Leu Lys Ala
755 760 765

Asn Ala Thr Glu Thr Leu Ala Gly Leu Arg Asn Asn Leu Thr Leu Gln
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Ile Asn Ala Glu Gln Ile Val Ile Lys Ile Thr Asp Gln Gly Tyr Val
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Thr Ser His Gly Asp His Tyr His Tyr Tyr Asn Gly Lys Val Pro Tyr
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Asp Ala Ile Ile Ser Glu Glu Leu Leu Met Lys Asp Pro Asn Tyr Gln
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Leu Lys Asp Ser Asp Ile Val Asn Glu Ile Lys Gly Gly Tyr Val Ile
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Lys Val Asn Gly Lys Tyr Tyr Val Tyr Leu Lys Asp Ala Ala His Ala
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